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CHEMOSYNTHETIC GAS EXCHANGER

FINAL REPORT

Period June 1963 through June 1964

ER 13270-4

JULY 1964

Prepared by the Martin Company

under

NASA Contract NASw-713

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Investigator: Leonard Bongers

FOREWORD

This Annual Report has been prepared by the Research Department of the Baltimore Division of the Martin Company in compliance with NASA Contract NASw-713, dated 4 June 1963.

The Martin Company acknowledges the support of NASA in conducting this investigation.

The work conducted under NASw-713 is being continued under a new NASA Contract (NASw-791) dated June 1964.

CONTENTS

I. INTRODUCTION

The hermetically sealed fish bowl where no matter can pass in or out, but in which both plant and fish can apparently exist indefinitely, is a simple example of the material balance at which we are aiming in developing life support systems for space exploration. To accomplish this feat, man's physiological requirements and limitations must be accurately known and techniques must be developed to cultivate autotrophic organisms which completely satisfy man's requirements.

Two types of organisms (plants) have been suggested to accomplish the objective of supporting man in a confined space: algae and hydrogen bacteria (Hydrogenomonas spp). Both reduce carbon dioxide via similar pathways into cell material; however, each utilizes energy from a different source. The algae--green plants--utilize light as an energy source for the reduction process. Hydrogen bacteria--colorless plants--obtain the energy needed for the assimilation of carbon dioxide by oxidizing gaseous hydrogen. This distinction is advantageous with respect to the specific application under consideration. Hydrogen and oxygen gases can be generated far more economically than visible light if only electricity is available (80% as opposed to 10% efficiency).

Consequently, the approach which employs the hydrogen bacteria for the synthetic process appears to hold more promise. Aside from the more efficient energy utilization (electrical energy to food energy), the weight and volume penalty imposed by the bacterial system is more compatible with space operations. Substantiation for this opinion is given elsewhere.[1, 2]

The system we are concerned with here is based on the use of electrolysis of water and bioregeneration by hydrogen bacteria. Electrolysis provides the oxygen for human respiration. The simultaneously evolved hydrogen and the exhaled carbon dioxide are converted into cell material (carbohydrates, fats, proteins, etc.), utilizing energy supplied by additional water electrolysis. The principle of operation of a life support system based on the coupling of electrolysis of water with biosynthesis by hydrogen bacteria is depicted in the flow diagram of Fig. 1. The material balance is illustrated in the chemical equations under the diagram. Food is symbolized by "CH_2O_2" and supplied at a rate of 2800 kilocalories per day. The oxygen requirement of man is estimated to be on the order of 22 liters per hour. The continuous energy input to electrolysis would be approximately one kilowatt per man. From the data discussed in this report, it appears that the proposed bacterial system is feasible for space application with respect to power and weight requisites.

CHEMOSYNTHETIC ECOSYSTEM

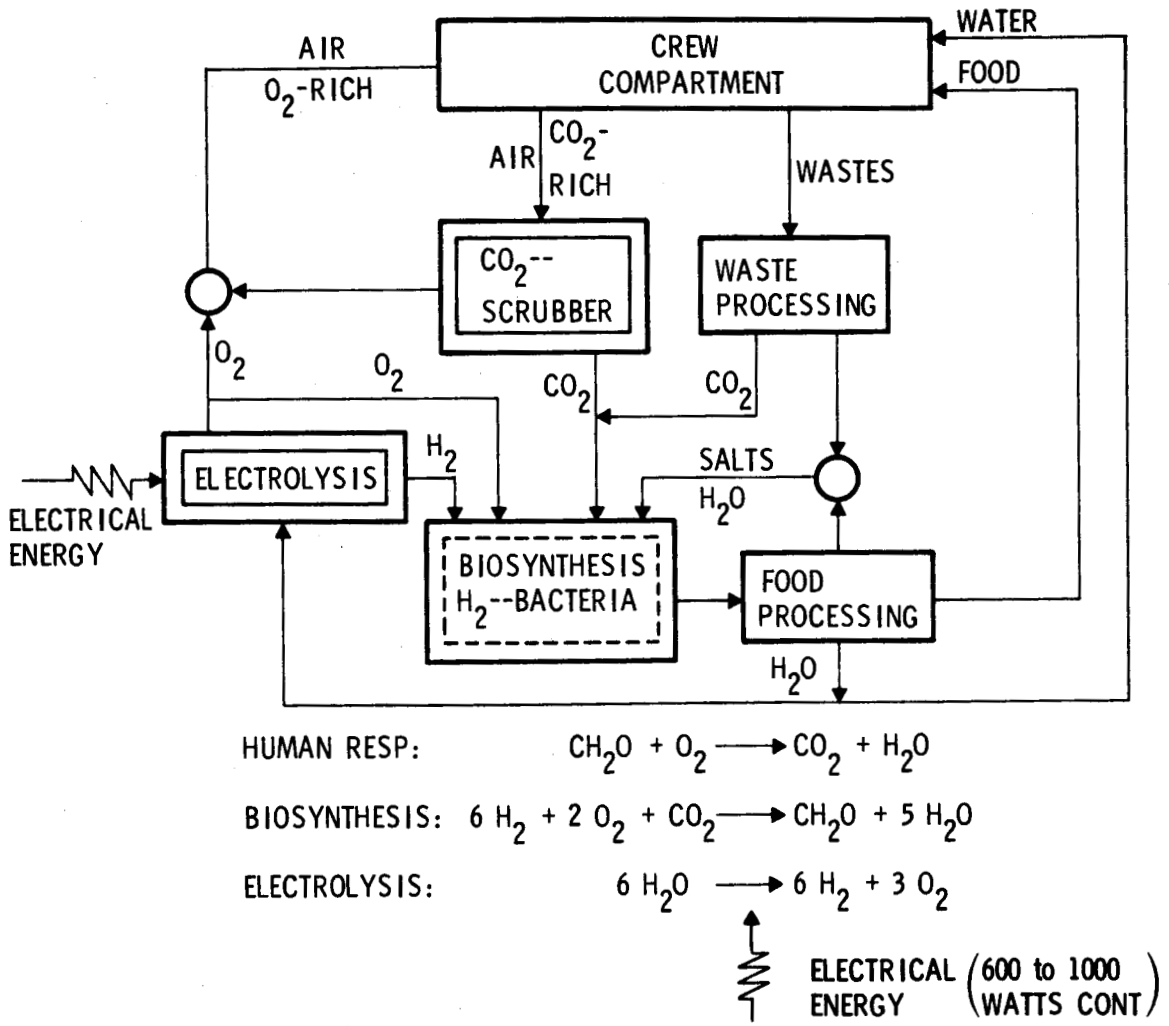

Fig. 1. Hypothetical flow diagram for an inboard bioregenerative life support system utilizing hydrogen and oxygen generated by the electrolysis of water. These gases in combination with the CO_2 and nitrogen excreted by the astronaut furnish substrate for bacterial growth. Bacteria, in turn, furnish human substrate.

In terms of growth rate and food production, nothing rivals microorganisms. As an example, a statement by Thaysen[3] is quite pointed. He says that a 1000-lb steer produces 0.9 lb of protein per 24 hours. Bean plants would produce the equivalent of some 80 lb. In an equal time, 1000 lb of yeast would make some 50 tons of protein. The great advantage of a food source based on microorganisms is also pointed out by Bunker[4]. However, no evidence exists to guarantee the acceptability of microbial food as a sole source of human nutrition. Further research is required to ascertain precisely the nutritional value of this material. A compromise may be necessary and is possible. Its acceptability may become complete with small amounts of additives. A spectrum of intermediate possibilities exists.

One solution offered is the use of an intermediary between plant and man which would nutritionally enhance the substrate. Myers and Brown[5] had certain reservations concerning the additional energy demands in algae-based systems. However, the relatively low energy input requirement of the bacterial system is a distinct advantage and could allow processing of some of the microbial product in this way. Presently, no basis exists for reliable estimates in this respect.

This first annual report presents results of investigations which had as main objectives the study of physiological growth requirements and limitations of Hydrogenomonas eutropha. The research Contract NASw-713 was directed toward the discovery of optimal conditions of growth of this organism in relatively densely populated, inorganic media. Growth characteristics studied were the effect of temperature, the formation of metabolic products, the efficiency of energy conversion and the effects of the gaseous and the inorganic substrates.

II. EFFECTS OF TEMPERATURE ON HYDROGENOMONAS EUTROPHA

Life processes are often very sensitive to changes in environmental temperature. Basically, life processes are chemical reactions, and part of the temperature sensitivity can be ascribed to the rates of chemical reactions. In this chapter, the temperature effects on growth rates and conversion efficiency of Hydrogenomonas eutropha will be discussed.

1. Materials and Methods

Media. The composition of the inorganic substrate used was, according to Repaske,[6] modified with respect to nitrogen source and final concentration of nitrogen and of some trace elements. The final concentrations used for these and other experiments (unless stated otherwise) were as follows: 0.025 M K-phosphate buffer, to which was added per liter, 10 ml of solutions containing per liter $CaCl_2 \cdot 2H_2O$, 10 gm; $CO(NH_2)_2$, 100 gm; NaCl, 10 gm; $NaHCO_3$, 100 gm; $MgSO_4 \cdot 7H_2O$, 10 gm; $Fe(NH_4)_2(SO_4)_2$, 0.8 gm; $CoCl_2 \cdot 6H_2O$, 2 mg; $MnCl_2 \cdot 4H_2O$, 40 mg; $CuSO_4 \cdot 7H_2O$, 20 mg; $Na_2MoO_4 \cdot 2H_2O$, 100 mg; $ZnSO_4 \cdot 7H_2O$, 100 mg; H_3BO_3, 30 mg. The starting pH of the medium was 6.0.

Gas mixture. The composition of the gases used for these experiments was 80% H_2, 10% CO_2 and 10% O_2. Total gas phase pressure was slightly above atmospheric pressure.

Measurements of growth rate. Cells were cultivated in 500 ml baffled Erlenmeyer flasks (containing 120 ml suspension each) which were placed in a thermostated rotary-type shaker. These flasks were connected in series with a 13-gal carboy, containing the gas mixture. A diaphragm circulation pump in the gas train ensured fresh supply of gas to the flasks.

The rate of growth could be determined in a number of ways: first, by measuring the optical density (650 mu) or dry weight content at the start and at the conclusion of an experiment. Optical density measurements could also be taken during an experiment. Gas uptake could be measured automatically and recorded throughout the time of an experiment. The system operated at an overpressure of 50 cm water. Pressure changes, the equivalent of 20 cm

of water, activated a pressure switch, which in turn opened and closed a gas inlet solenoid. If the pressure inside the flasks, due to gas consumption by the bacteria, decreased to approximately 20 cm (H_2O) overpressure, the pressure switch opened the gas inlet solenoid long enough for the pressure to build up again to approximately 40 cm.

This operation--"refill"--was illustrated by a digital counter. Because the total volume and temperature was kept constant, the volume of gas consumed during the experiment could be calculated. The digital summation counter used was photographed at half hour intervals by a simple, single frame, motion picture camera, and from the developed film, the rate of gas uptake could be calculated.

In order to maintain the composition of the gas mixture in the flasks identical to that in the gas storage reservoir, frequent equilibration of the gas in culture flask and reservoir was obtained by opening both the gas inlet and the gas outlet solenoids. If both solenoids are opened, the gas mixture of the large reservoir circulates through the flasks. This "wash time," triggered by a step relay, was preset to occur for one minute after five refills.

The time involved in each experiment depended on the temperature used. Temperatures tested ranged from 20° to 42° C. The results of conversion efficiency measurements will be presented here. Discussion of the apparatus used (Fig. 4) will be deferred to a later chapter.

2. Results and Discussion

a. Effect of temperature on growth and metabolic rate

Hydrogen bacteria were cultivated at various temperatures ranging from 20° C to 42.5° C. Growth rates were measured at temperature intervals of 2.5° C. The rate of growth was recorded as the total volume of gas taken up per unit of time. This procedure was adopted after it was observed that a constant ratio existed between the rate of increase in optical density of the suspension and the rate of gas uptake. This procedure is valid only under the conditions used for these experiments. Two methods of measurement were possible. First, uniformly dense cultures are permitted to grow at various temperatures for a selected length of time and their final population measured. Second, uniformly dense cultures are permitted to grow at various temperatures until a preselected population density is reached and the time interval is measured. Since the second method obviates substrate differences, it was the one used.

To eliminate the effects of growth rates being limited by oxygen diffusion, the maximal rate of oxygen transfer was accurately determined by the sulfite oxidation method.[7] It was found from these studies that at 35° C a suspension of less than 1.2 O.D. units (the equivalent of 0.4 grams dry weight per liter) received sufficient oxygen. Suspensions with a density in excess of this value are oxygen limited at this temperature, and, consequently, the growth rate is limited by oxygen supply rather than by temperature. The time (in hours) required for a fixed cell suspension to attain a density of 0.4, 0.2, or 0.1 was measured for each temperature tested (see Fig. 2).

The growth rate was found to be low at either temperature extreme. The time required to attain a standard density from a fixed initial density was much less at optimal temperature than at the temperature limits investigated.

All experiments shown in Fig. 2 were initiated at the same low density of 0.03 grams/liter, calculated from optical density measurements. Thirty-five degrees centigrade is shown to be the optimal temperature for growth of this organism. As can be seen in the figure, a density of cells which gives a dry weight of 0.2 gram/liter is obtained after about eight hours of growth at 35° C. At this density the logarithmic growth rate has stopped and linearity is maintained until the rate of oxygen diffusion becomes limiting. From this point on, no meaningful data relating temperature to growth rate are obtainable.

Growth infers metabolic activity, but the metabolic rates need not be in absolute synchronism with growth rates. To distinguish between these different aspects of biological activity, a number of short-term experiments were performed which measured the rate of metabolism (the rate of gas uptake) being considered as a sufficient criterion during twenty-minute periods, and at various temperatures between 15° and 48° C. These results are plotted in Fig. 3 (open circles) and are superimposed on data derived from Fig. 2. Several differences are evident. The optimal temperature for growth is 6° to 8° C lower than that for gas uptake. The rate of growth at 45° C is essentially zero, while gas consumption is still vigorous. Furthermore, although the rate of gas uptake at these elevated temperatures is high, it is unstable--falling rapidly after 40 to 60 minutes. High rate gas uptake is also observed at 48° C, but is even less stable. The experiments indicate that a relatively high rate of gas consumption can be sustained for a short time only and thus for practical purposes the strain examined here functions best at 35° C.

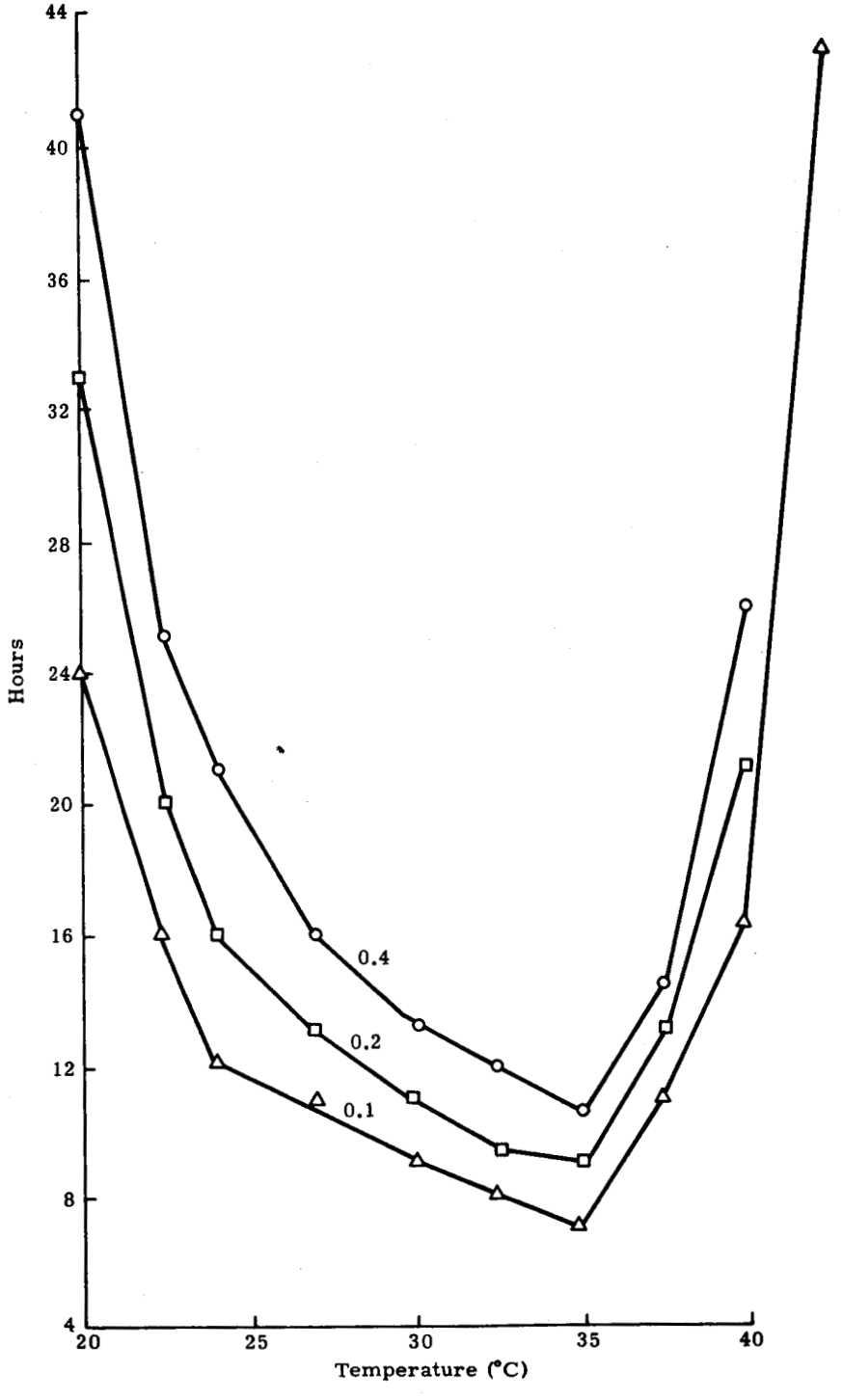

Fig. 2. Temperature effects on cell production. Times were measured for a set of predetermined cell densities (0.1 to 0.4 grams/liter) to be achieved by cultures incubated at various temperatures, from a fixed initial cell concentration. Hydrogenomonas eutropha. Gas Phase: 80% H_2, 10% O_2, 10% CO_2.

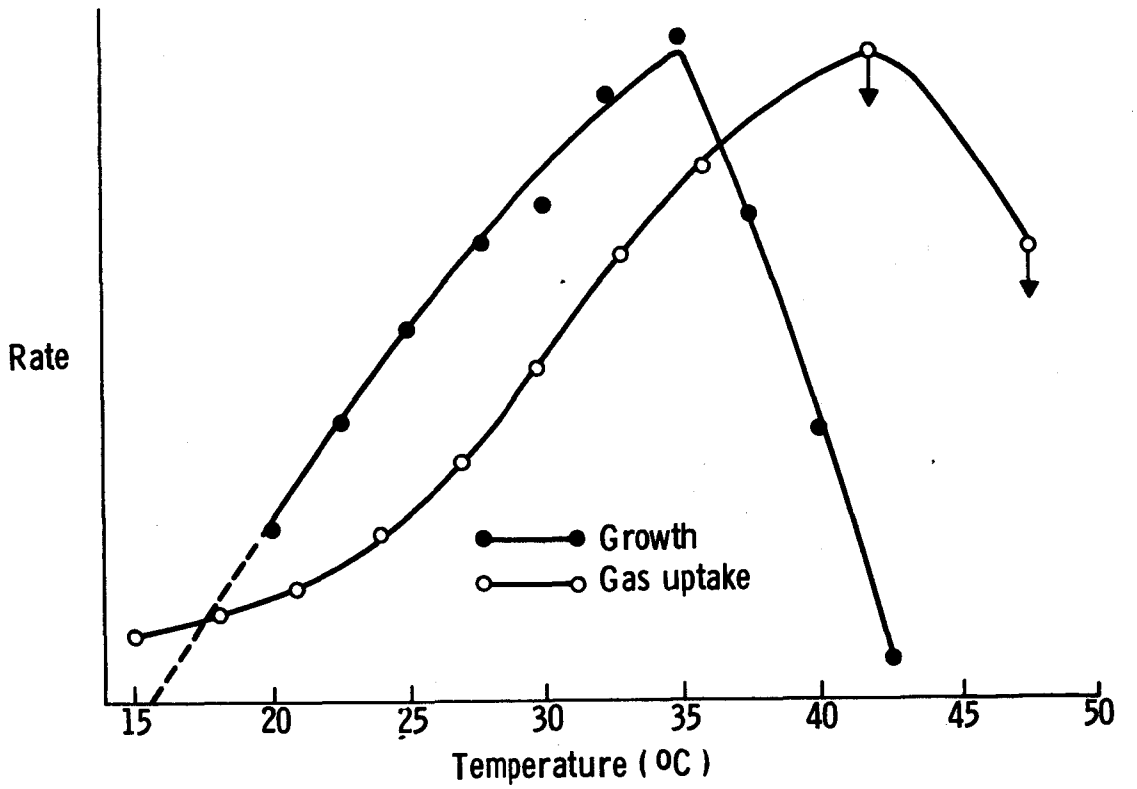

Fig. 3. Relationship between gas uptake and temperature. The growth versus temperature curve calculated from data in Fig. 2. Hydrogenomonas eutropha. Gas phase: 80% H_2, 10% O_2, 10% CO_2.

b. Effect of temperature on the efficiency of energy utilization

The power required to impel the proposed chemosynthetic bioregenerative system presents itself as the second major aspect of our studies. Carbon fixation occurs in reaction (1):

$$E + 2H_2 + CO_2 \longrightarrow CH_2O + H_2O \qquad (1)$$

This reaction requires energy (E). This energy requirement is furnished by reaction (2).

$$N/2\ (2H_2 + O_2 \longrightarrow 2H_2O + E) \qquad (2)$$

Since, thermodynamically, each carbon fixed requires a certain energy expenditure, and each hydrogen burned represents a certain amount of energy produced, the ratio of hydrogen molecules (N) consumed for each CH_2O produced is a measure of the efficiency of energy conversion.

To determine conversion efficiency as a function of temperature, the value for N was found at 25° and 35° C in the following fashion: The amount of CO_2 fixation was determined from the carbon content (measured with C-H analyzer) and from the increase in cellular mass during the course of the experiment. The amount of hydrogen consumed in reaction (2) was found by subtracting the volume of gas consumed in reaction (1) from the total gas volume consumed.

Previous observations [8] have suggested that the average value of N is 4 to 5. Table 1 contains data obtained at both optimal and suboptimal temperatures. These data indicate that there are no significant temperature effects upon conversion efficiency.

TABLE 1

Conversion efficiency (expressed as N, see text) at 25° C and 35° C, and specific conversion rate (expressed as milliliters of CO_2 assimilated per hour per gram of dry weight); Hydrogenomonas eutropha; Gas phase: 80% H_2, 10% O_2, 10% CO_2

Number of Experiments	Temperature (°C)	N	Average Specific Rate
13	35	4.9 ± 0.2	127
10	25	5.6 ± 0.5	85

III. EFFECT OF GASEOUS SUBSTRATE ON THE GROWTH OF HYDROGENOMONAS EUTROPHA

Hydrogenomonas eutropha requires three gases (CO_2, H_2 and O_2) for autotropic growth. Under optimal conditions, these organisms consume the gaseous substrate in the following molar ratios:

$$6H_2 + 2O_2 + CO_2 \longrightarrow CH_2O + 5H_2O.$$

Experiments were conducted to determine the optimal concentrations of these gases with respect to growth.

1. Materials and Methods

The culture assembly*, shown in the diagram of Fig. 4, was constructed so as to obtain a high rate of gas transfer. The reaction vessel (R. V.) which has a total volume of approximately 200 ml, contained 20 to 50 ml of cell suspension. In order to maintain a constant total pressure on the system pressure, a switch-solenoid assembly was connected to the shut-off valve 4, which was preset at the desired pressure. Using this apparatus, suspensions of 50 optical density units (approximately 15 grams dry weight per liter) or less were examined.

With relatively dense suspensions, the rate of gas uptake is so high that no accurate reading on the manometer (M) could be made. Two means were used to decrease the extreme manometric changes. First, the ratio of liquid to gas phase was changed by inserting a 250-ml compensation vessel in the gas train, and, secondly, a manometer fluid with a specific gravity of 2.9 was used. In order to prevent evaporation in the R. V., the gas stream was humidified in the wash bottle (W. B.).

The rate of gas flow through the suspension and the wash bottle could be regulated with the needle valves 6 and 5, respectively. All three containers, the reaction vessel, the compensation vessel, and the wash bottle, were submerged in a constant temperature (35° C) water bath during the experiments.

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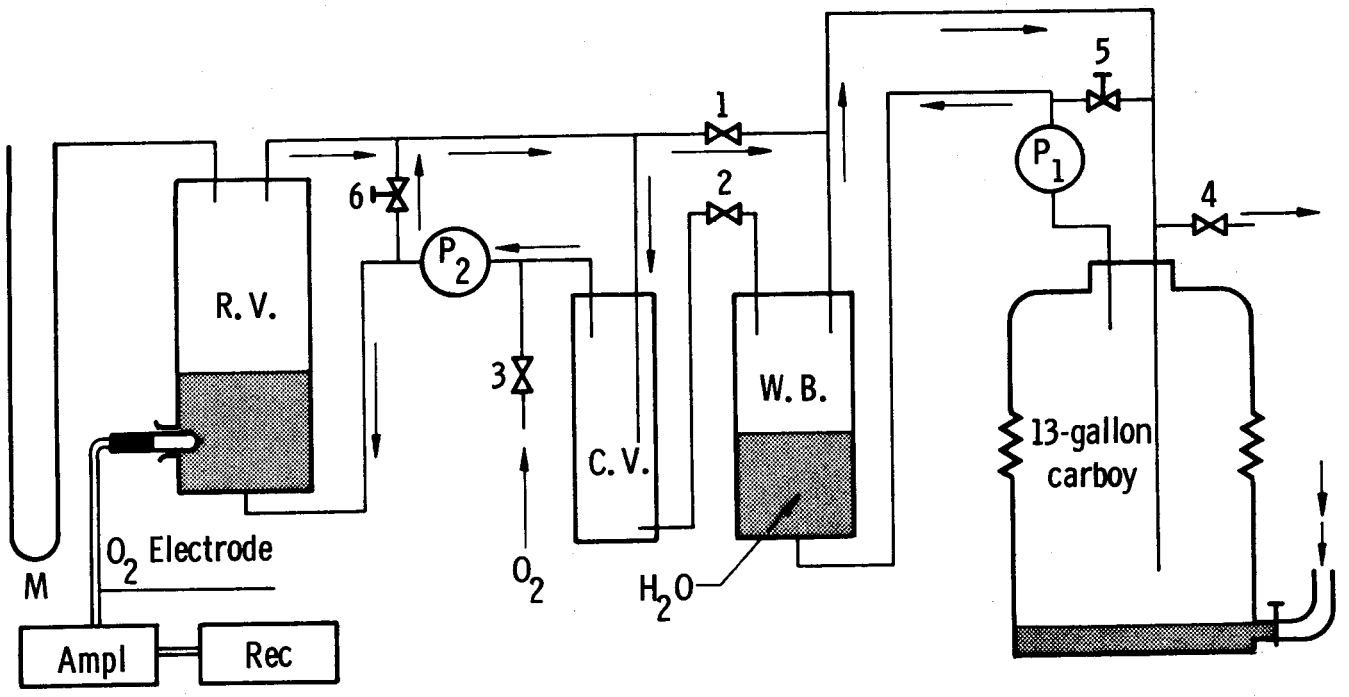
* A simplified version of this apparatus was used during earlier experiments. The oxygen sensor was not available at that time.



Needle valve

Shut-off valve

$P_{1, 2}$ Diaphragm-pump

R.V.: Reaction vessel with suspension

C.V.: Compensation vessel (250 ml)

W.B.: Wash bottle

M: Manometer

Fig. 4. Culture Assembly: The schema of the device used to measure gas consumption of a culture of Hydrogenomonas eutropha under the condition controlled dissolved oxygen concentration. Single arrows indicate direction of gas flow. Double arrow indicates direction of water flow. Other symbols as indicated.

Valves 1 and 2 were always open except when gas uptake was measured. When these valves were closed, the gas pressure in the reaction vessel and the compensation vessel decreased due to gas consumption by the cells. The rate of gas consumption was measured using the manometer (M). From the change in pressure with time, the absolute amount of gas taken up was calculated. If conversion efficiency measurements were desired, the suspension was sampled for dry weight at the start and at the conclusion of the experiment, and the samples were analyzed for carbon content with a carbon-hydrogen analyzer (Coleman 29). In addition, gas uptake measurements were made at frequent intervals during the experiment, and the total gas consumption calculated. From the carbon fixation and the total gas consumption, the efficiency was calculated.

A diaphragm recirculation pump (P2) forced the gas mixture through a sintered glass filter (medium porosity) situated at the bottom of the reaction chamber. Oxygen was measured by means of a probe (Beckman) which was fitted into the reaction chamber. This oxygen sensor and accessory equipment indicated the oxygen concentration in the liquid phase, and maintained automatically a preset dissolved oxygen concentration by increasing the oxygen partial pressure in the gas phase. This consisted of a probe amplifier, recorder, and a solenoid assembly which was connected between the oxygen cylinder and the shut-off valve 3.

Experiments were carried out at 35° C (unless stated otherwise), the optimal growth temperature for Hydrogenomonas eutropha, used in this series of experiments.

For dry weight determination, the cells were separated from the medium by centrifugation, washed in H_2O, and subsequently dried for 24 hours at 60° C under vacuum.

Optical density measurements were made in a Beckman DK spectrophotometer at 650 mu. Heavy suspensions were diluted to measurable cell densities. A fixed relation between turbidity and dry weight of cells was shown to exist for cells of normal composition. An optical density value of 1.0 indicates a dry weight content on the order of 0.3 to 0.4 grams per liter. For cells cultivated under conditions of nitrogen starvation, the value increased and reached 0.5 to 0.6 grams per liter (also see Schlegel [9]).

Fat inclusions were analyzed by a procedure adopted from Williamson, et al.[10] The concentration of the lysed material was measured at 650 mu with the spectrophotometer. The relationship between optical density and dry weight was determined to be on the order of 0.30 to 0.35 grams dry material per liter for one optical density unit. Standardization of the procedure for cell lysing was found to be critical for accurate measurements.

2. Results and Discussion

A series of experiments were carried out to examine the toleration of Hydrogenomonas eutropha to various concentrations of carbon dioxide, hydrogen and oxygen. The objectives of these studies were: (1) to determine to what extent relatively high carbon dioxide concentrations can be utilized to effectively prevent pH increases, (2) to determine if there is any effect of low hydrogen partial pressure on the growth rate of hydrogen bacteria. (The use of relatively lower hydrogen partial pressure would result from the increase in the carbon dioxide supply), and (3) to determine the effect of oxygen on growth rate, conversion efficiency, and chemical composition of the bacteria.

These studies were done at a total gas pressure of approximately one atmosphere. Molecular nitrogen gas was used as balance.

a. Effect of hydrogen and carbon dioxide concentrations on growth

The oxygen content of the gas mixture was held constant at 10% for experiments in which hydrogen and carbon dioxide concentrations were varied. The experiments were maintained for 5 to 6 hours, during which time the increase in population density was measured.

Final cell concentrations obtained were on the average 5 to 6 times the initial values with 5 to 25% carbon dioxide in the gas phase. (Suspensions were started at relatively low optical density values, on the order of 1.5 to 2.5, to ensure adequate gas supply.) Somewhat lower values were found (3 to 4 times) at 60% carbon dioxide in the gas phase. In the latter case, the suppression of the reproduction might be substantially due to a decrease in pH.

When hydrogen concentrations were varied from 5% to 80%, the growth rates obtained were substantially identical.

b. Effect of oxygen concentration on growth

The effect of oxygen supply on the growth rate of Hydrogenomonas eutropha was described earlier (2, 6, 8). This important factor and its effect on the growth of this organism was re-investigated, utilizing relatively light suspensions and a wide range of oxygen concentrations. More accurately controlled conditions with respect to oxygen supply (see Figs. 6 and 7) could be obtained with the use of the macrorespirometer with the oxygen sensor depicted in Fig. 4.

The results obtained, recorded in Table 2, essentially confirmed previous observations.[8]

TABLE 2

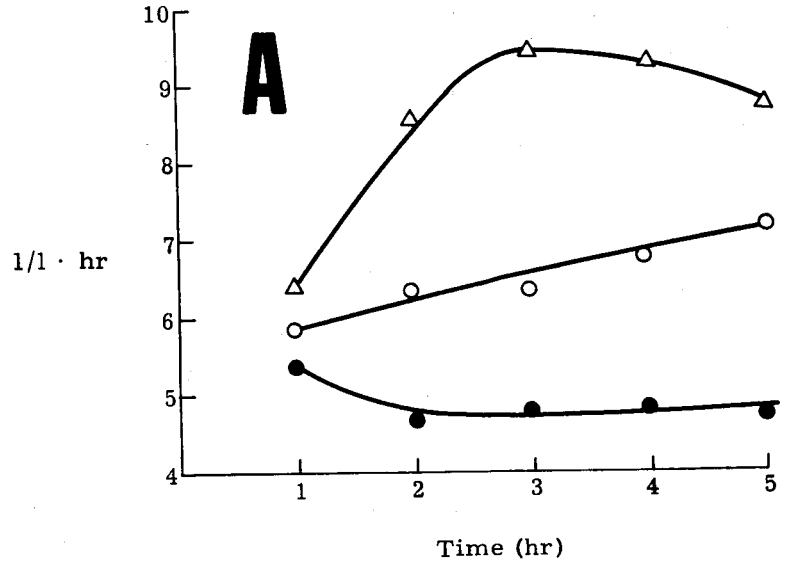
Effect of O_2 on Growth of Hydrogenomonas eutropha. Gas phase: 70% H_2; 10% CO_2; % O_2 as indicated; (N_2 balance). The temperature was maintained at 35° C. The average Q_{CO_2} was calculated as one-ninth of total gas uptake (see Eqs. (1) and (2)). Starting density was 2 grams per liter.

% O_2	5	10	15	20
Q_{CO_2}	183 ± 9	185 ± 9	154 ± 9	139 ± 7
Rel. Val. (%)	100	101	84	76

Similar results are illustrated in Fig. 5 A, B. With 10% O_2 in the gas phase, the rate of (total gas) uptake increases with time, due to the increase in cell concentration (see Fig. 5A). Only a slight increase is observed with 20% O_2 in the gas phase. With 30% oxygen in the gas phase, an initial decline in rate of gas consumption is followed by a rate which remains constant. Apparently, cell activity is severely inhibited under this condition.

The specific rate of gas uptake (H_2 + O_2 + CO_2) declines under all conditions tested. The cause for this decline has different origins. The decline with 20 and 30% oxygen can mainly be ascribed to oxygen inhibition. The decline in specific rate with 10% oxygen is mainly due to the fact that fat formation occurs (discussed later). Presumably, the oxygen supply becomes limiting, which induces the formation of storage material, a cellular constituent which contributes to dry weight, but which is inactive with respect to cell reproduction.

Due to uncertainties with respect to the availability of oxygen to the organisms, attempts were made to measure the actual oxygen concentration in solution. The Beckman oxygen sensor (see Fig. 4) provided this information. The sensor, calibrated at the start and conclusion of an experiment, was sufficiently stable for this purpose.



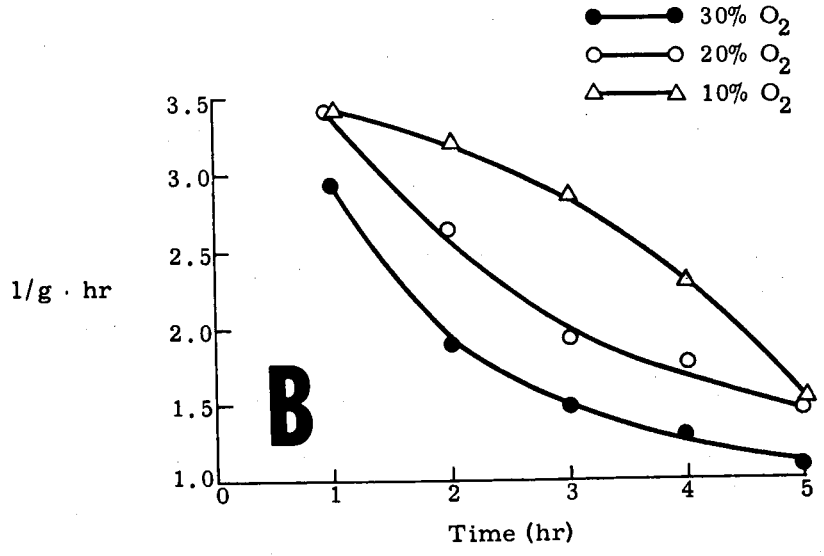


Fig. 5. Gas uptake by Hydrogenomonas eutropha, with 10, 20 or 30% O_2, 60% H_2, and 10% CO_2 in gas phase (N_2 balance). Starting dry weight: approximately 2 grams per liter. Temperature: 35° C.

A. Uptake of (H_2 + O_2 + CO_2) in liters, per liter of suspension, per hour versus time in hours.

B. Specific rate of gas uptake, expressed in liters, per gram dry weight, per hour versus time in hours.

The dissolved oxygen concentration desired for a particular condition was maintained throughout the time of experiment. The oxygen concentration at the start of the experiment was sufficient to meet the demand of the suspension. If, due to increase in cell concentration, the oxygen consumption increased, pure oxygen was added to the gas mixture and, after equilibration, the excess pressure vented. The rate of gas flow was kept constant during the course of the experiment. With this procedure, the oxygen content of the gas phase increases according to the demand set by the rate of oxygen consumption by the cells. As the gas flow rate was constant, the diffusion rate increased proportionally with oxygen concentration. However, the diffusion rate for carbon dioxide and hydrogen decreases at the same time. In order to prevent CO_2 limitation, the carbon dioxide content of the initial mixture was increased to 15%. The hydrogen content of the starting mixture varied from 65 to 75% in the gas phase. As discussed earlier, there is no reason to expect the hydrogen supply to become rate limiting.

The results of experiments related to oxygen concentration in the liquid phase are plotted in Figs. 6 and 7. The specific rate of CO_2 fixation (Q_{CO_2}) and the rate of CO_2 fixation per liter of suspension is highest at the lowest oxygen concentration tested (0.05 mM; this is the dissolved oxygen concentration which would result if approximately 5% O_2 was present in the gas phase, at atmospheric pressure, and under conditions of gas equilibration). These relatively high initial rates confirm that the lower oxygen concentration, the higher is the rate of cell metabolism. In other words, a relatively low oxygen concentration has an inhibiting effect on the growth of Hydrogenomonas eutropha. Of course, some "critical" oxygen concentration is required.[11,12] This critical level of oxygen concentration in solution is not determined. For practical purposes, relatively higher oxygen levels are necessary for both maximum cell formation and to avoid metabolic transition to inactive resting cells (with its accompanying high rate of storage material accumulation). This transition from active into inactive cell metabolism is evident in Fig. 6b. The initial rate of gas uptake, with oxygen concentration levels of 0.05 and 0.08 mM, declines after 1 and 2 hours, respectively, to considerably lower values. After 5 hours of growth, the same trend can be observed at an oxygen level of 0.13 mM. It is, however, not certain whether this is to be attributed to the same phenomenon. Depletion of constituents in the inorganic substrate might have interfered. At any case, no decline is evident at an oxygen level of 0.19 mM. However, the total mass of cells formed under this condition (see Fig. 7) is considerably less than with 0.13 mM oxygen in the liquid phase.

From experiments described, one may conclude that excess oxygen inhibits growth. The mechanism of inhibition is not well understood. The inhibition is attributed to the interaction of oxygen with hydrogenase, a phenomenon observed by Fisher, et al.,[14] with cell-free extracts of P. vulgaris. However, we found

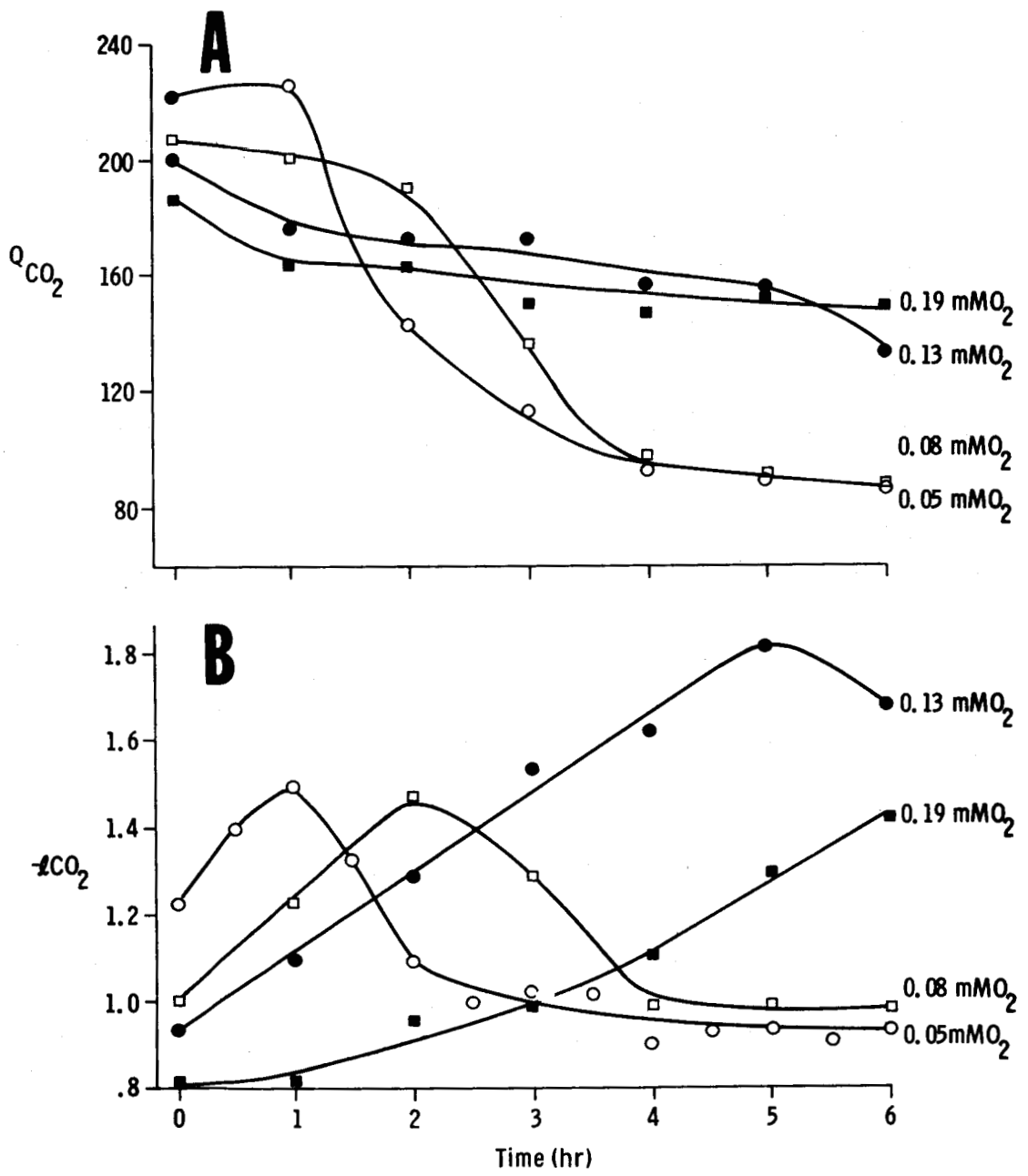


Fig. 6. The rate of carbon dioxide fixation as a function of time by a culture of Hydrogenomonas eutropha at four different maintained concentrations of solvated oxygen.

A. Q_{CO_2} is equal to the rate of CO_2 uptake (in milliliters) per hour by a milligram dry weight of cells.

B. The amount (in liters) of CO_2 at STP taken up per hour by one liter of cell suspension.

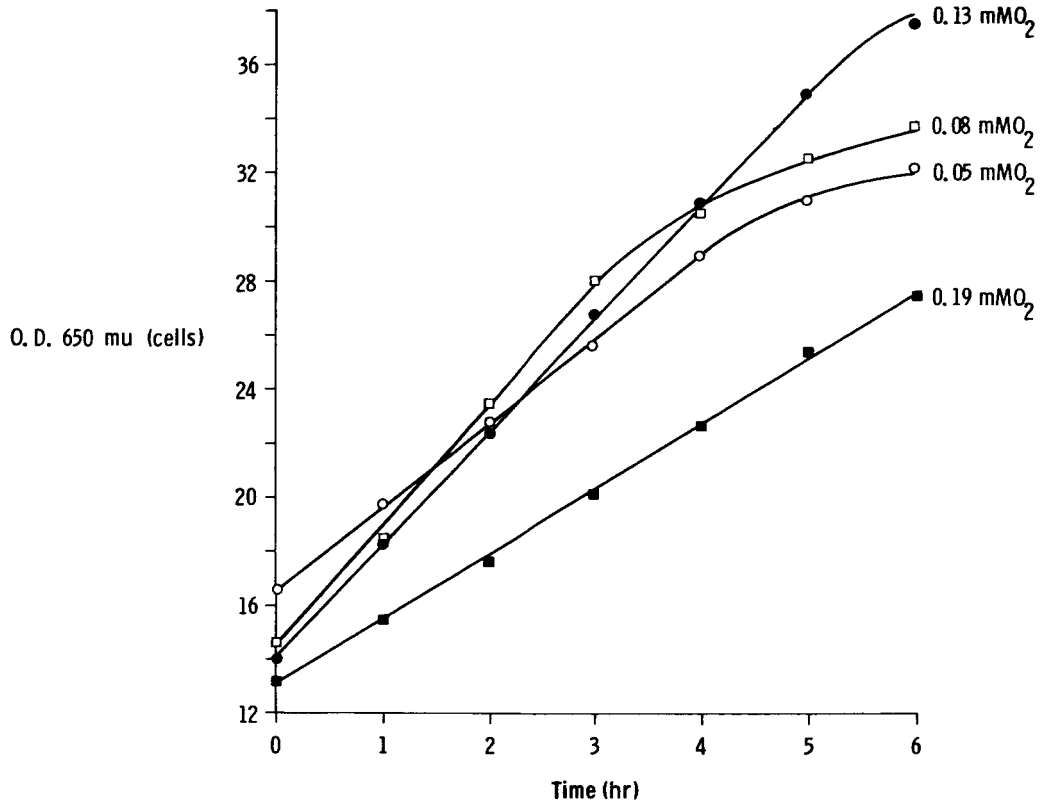


Fig. 7. The increase in optical density (measured at 650 mu) of four cultures of Hydrogenomonas eutropha cells plotted against time. Each of the four suspensions was grown at the indicated concentration of solvated oxygen.

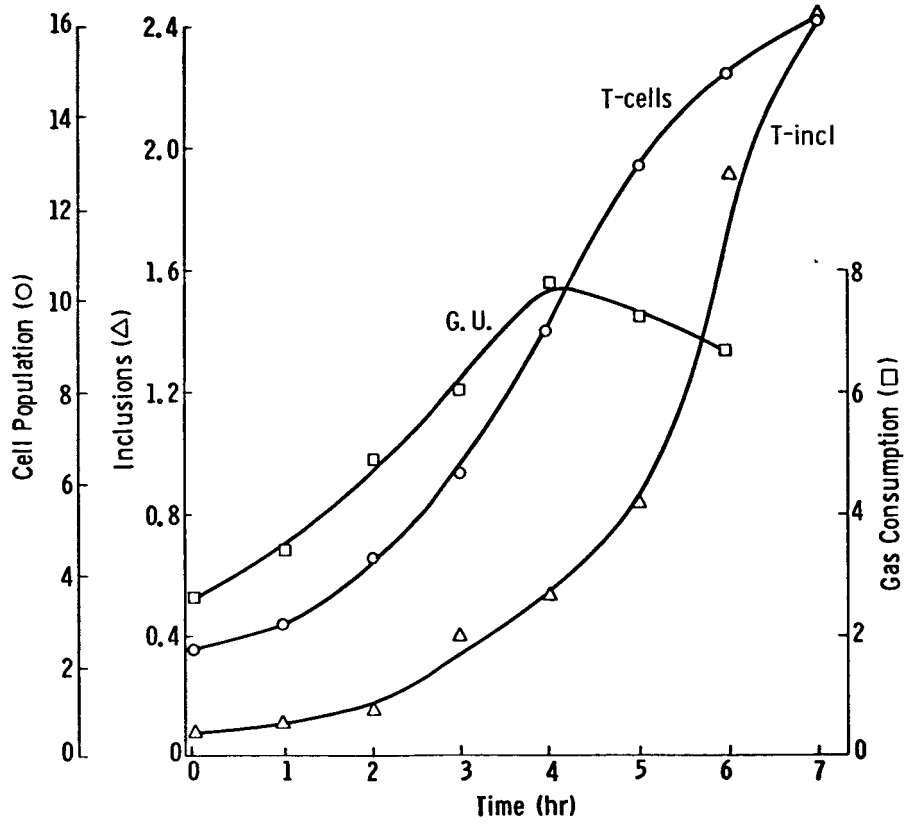


Fig. 8. The change in three culture characteristics as the critical transition in oxygen concentration from optimal to suboptimal occurs. Cell population is based on culture densities measured at 650 mu. Inclusions (globules of poly-β-hydroxybutyrate) are obtained by cell lysis in a sodium hypochlorite solution and the resulting suspension measured at 650 mu. Gas consumption in liters per hour per liter of suspension.

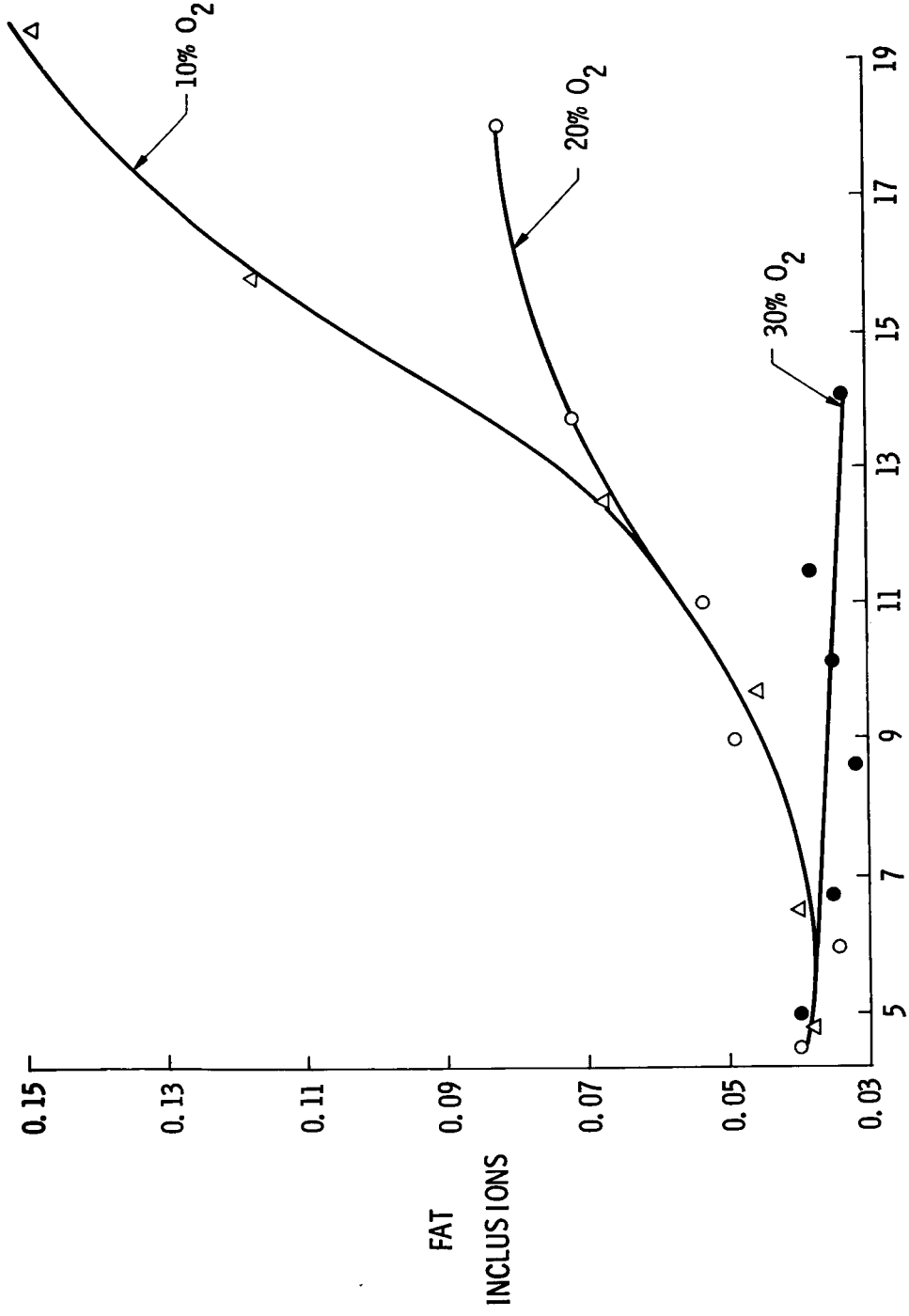


Fig. 9. The formation of "fatty" inclusions as a function of oxygen supply and cell concentration.

Ordinate: ratio of OD inclusions over OD cells
Abscissa: cell concentration in OD units
Conditions as in Fig. 8.

no inhibition in the rate of hydrogen oxidation with gas mixture containing 5 to 50% oxygen in hydrogen. Equal rates of hydrogen oxidation were observed at these oxygen concentrations over a reaction time of 90 minutes. At all concentrations tested, a slight decrease in rate was observed toward the conclusion of the experiments, but no severe inhibition of the hydrogen-oxidation system was found to occur.

Low oxygen concentrations are favorable with respect to gas uptake, but cell metabolism leads to the formation of inactive cells, which is a disadvantage in case of continuous cultivation. With respect to mass production of cells, the level of oxygen concentration must be on the order of 0.13 to 0.19 mM.

c. Effect of oxygen on the cell composition

According to Schlegal,[13] gas limitation leads to the formation of fat-like inclusions in hydrogen bacteria. In our experiments no evidence of fat formation was found with relatively low hydrogen and low carbon dioxide concentrations. A sub-optimal oxygen supply had a pronounced effect, however, on the chemical composition of the resultant cell mass. Under these conditions, an energy-rich storage polymer is produced consisting mainly of poly-β-hydroxybutyric acid.

Figure 8 illustrates the effects which accompany the transition from optimal to sub-optimal oxygen supply. Three measurements were made throughout the course of the experiment: the rate of gas uptake (G.U.), the increase in population density (T-cells), and the formation of cellular inclusions (T-incl). It can be seen in this figure that under optimal conditions (0 to about 3 hours) the cell population increases almost logarithmically, and that the gas uptake increases more or less proportionally with suspension turbidity. During this phase the formation of inclusions is relatively low. In the next two hours, a gradual change in cell activity takes place. In the final three to five hours, cell metabolism is apparently directed toward the formation of this fat-like material. This change in metabolic activity is evident in the rate of gas uptake and the rate of increase in cell population. The rate of gas uptake becomes either constant or levels off, and the rate of population increase usually declines.

No gassing conditions, other than that of sub-optimal oxygen supply, were expected to initiate the production of the storage material. To verify this assumption, hydrogen bacteria were cultivated with gas mixtures with oxygen concentrations relatively higher than used before (20 and 30%O_2 in gas phase). The effects of the higher concentrations of oxygen on cell metabolism are illustrated in Fig. 9. At 30%O_2, cell multiplication is lower, but no inclusions are formed. Also at 20%O_2 the formation of inclusions is considerably less compared to suspensions with 10%O_2 in the gas phase. It appears from these observations that oxygen impoverishment induces hydrogen bacteria to change their metabolic activity toward the formation of poly-β-hydroxybutyrate.

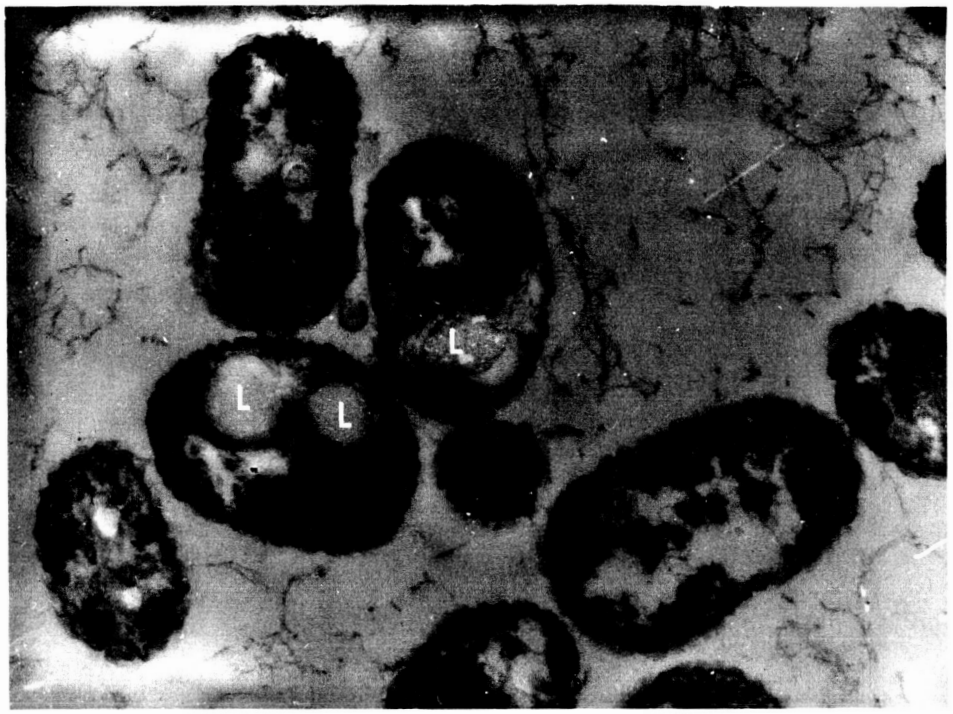


Fig. 10. Electron micrograph of Hydrogenomonas eutropha cells. L: fatty inclusion.

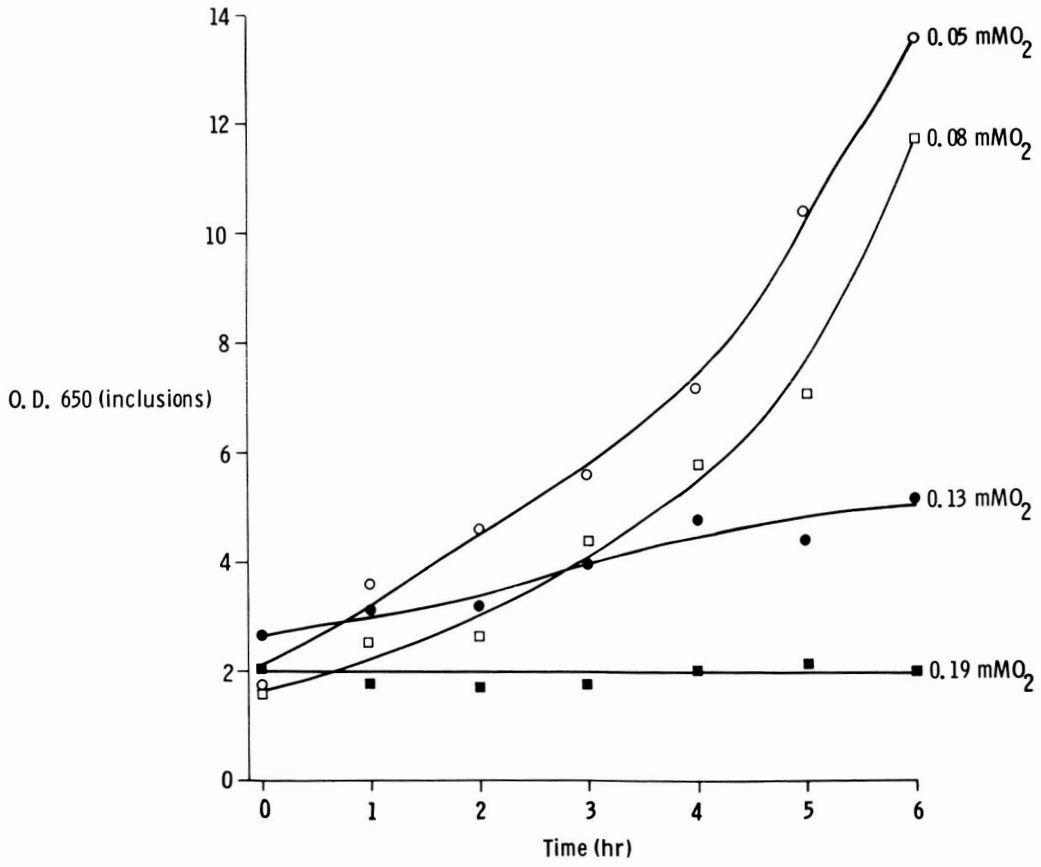


Fig. 11. The production of poly-β-hydroxybutyric acid containing inclusions in culture of Hydrogenomonas eutropha as a function of time. A fixed concentration of solvated oxygen was maintained in each of four cultures as shown. The cells were hydrolyzed in a solution of sodium hypochlorite and the resultant suspension of inclusions measured at 650 mu.

Cells accumulating relatively large amounts of this storage material cease actively dividing. In the electron micrograph of Fig. 10, the globular inclusions (L) are distinguishable in the cytoplasmic material.

In order to determine the exact oxygen concentration which inhibits the formation of the storage polymer, a series of experiments were conducted in which the solvated oxygen concentration was monitored and maintained at a given level throughout the time of experiment. The gas mixture used for these experiments was enriched in oxygen as required, which was determined by the oxygen sensor (the macro-respirometer depicted in Fig. 4 was used for these experiments). This technique was used to maintain the dissolved oxygen concentration at the desired level. Four oxygen concentrations were tested: 0.05, 0.08, 0.13 and 0.19 mM of dissolved oxygen per liter. The results of these experiments are plotted in Fig. 11. These experiments confirm the earlier mentioned effect of oxygen on the formation of the storage polymer, and also, that oxygen concentration in excess of 0.13 mM, inhibits the formation of this polymer.

d. Effect of oxygen concentration on the efficiency of energy utilization

As discussed in the previous paragraphs, the oxygen concentration has a profound effect upon the growth rate of H. eutropha. It was also shown that the composition of the formed product(s) depends on the conditions of oxygen supply. Also, the efficiency of energy conversion is affected by the oxygen concentration, to which the cells are subjected.

The "biological efficiency"--defined in Section II-2 (see also Ref. 1) as the number (N) of hydrogen molecules combusted for each molecule of carbon dioxide assimilated by the cells--may vary considerably. The best values for N reported [15] show a combustion of two hydrogen for the fixation of one CO_2. Average values for N indicate the combustion of 4 molecules of hydrogen.[15,16,17] For further discussion with respect to the energetics of carbon dioxide fixation, the reader is referred to publications in this field.[18,19]

For measurements of the effect of oxygen upon conversion efficiency of H. eutropha, cell suspensions were cultivated in a macrorespirometer, similar to the one depicted in Fig. 4, except that no oxygen sensor was used. Precise measurements of the dissolved oxygen concentration could therefore not be made. The oxygen content of gas mixture tested with respect to their effect on efficiency ranged from $5\%O_2$ to $20\%O_2$. The results of a number of experiments are recorded in Table 3.

TABLE 3

Oxygen supply as related to the conversion efficiency in H. eutropha. Gas phase contained 70%H_2, 10%CO_2, and oxygen as indicated. Time of experiment was 4 hours; the temperature 35° C.

%O_2	5	10	15	20
N	3.9 ± 0.3	4.2 ± 0.2	5.7 ± 0.3	6.0 ± 0.4

It is evident from these results that "excess" oxygen supply leads to conditions which are relatively less efficient with respect to energy conversion. A further qualification is necessary. The procedure of analysis is based on measurements of total gas uptake and the total fixation of carbon. If the material produced under low oxygen supply (5 and 10%O_2) is different from the organic material obtained with gas mixture of high oxygen content (15 and 20%), the difference in energy expenditure can then be attributed to difference in cell composition. Another complicating phenomenon is that oxygen impoverishment leads to the formation of resting cells (discussed previously). Cell division ceases under these conditions and consequently energy expenditure is less.

The preliminary conclusions which may be drawn from these experiments are that the metabolic activity which leads to the formation of resting cells--cells relatively poor in protein and rich in storage material--is an efficient process, and that the so-called "idling" is not enhanced by this condition.

3. Conclusions

The experiments described above do permit certain conclusions with respect to the operation of an ecosystem based on hydrogen bacteria. The concentration of dissolved oxygen plays an important role with respect to growth rate, the efficiency of energy conversion and the characteristics of the produced material.

If the oxygen supply leads to solvated oxygen concentrations in excess of approximately 0.15 mM, the production of cell mass is temporarily lower than with lower oxygen concentrations. If the oxygen concentration is maintained well below 0.10 mM, the produced cell material becomes gradually less active with respect to gas uptake and cell multiplication and, consequently, less cell mass will be produced in the long run. For continuous operation, it seems, therefore, that solvated oxygen concentrations on the order of 0.13 to 0.19 mM are required. To produce material which is relatively rich in storage products (and relatively poor in protein), dissolved oxygen concentrations well below 0.10 mM are implied.

The electrical input (into water electrolysis) to drive the biosynthetic process depends upon the desired chemical composition of the product. To produce material which contains a high content of the storage polymer, less energy is required than to maintain actively reproducing protein-rich cells.

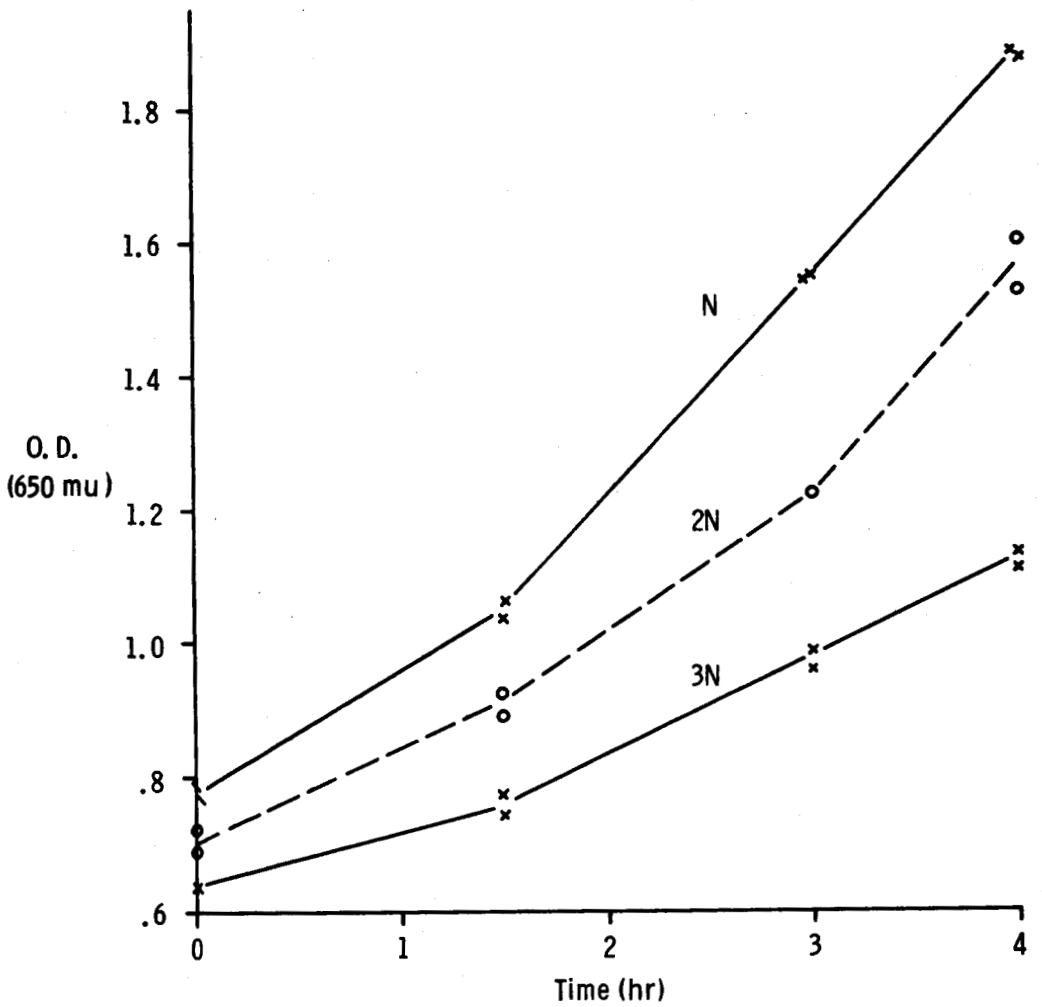


Fig. 12. The effect of medium concentration on cell density (measured spectrophotometrically at 680 mu) plotted as a function of time. Growth kinetics on basic medium (N) are compared to activity on multiples of this basic medium (2N) and (3N).

IV. THE INORGANIC SUBSTRATE

The Hydrogenomonads are autotrophic organisms which require a mixture of three gases and a solution consisting of inorganic salts as a substrate for growth. To grow efficiently, each cell must receive the necessary amount of each of the required elements of its substrate. The gases are usually separated and can be fed to the gas mixture whenever the monitoring probes indicate that their partial pressure in solution falls below optimal (see above). The concentration of the inorganic salts in the medium is less easily controlled.

1. Cell Tolerance to Osmotic Pressure as Examined with Multiples of the Basic Medium

Since the concentrations of each ionic species used in the substrate were arrived at by the simple expedience of having the cells grow--that is, cell growth could tolerate the presence of these salts in these amounts--the foundation for quantitative demands by the cells does not exist.

The initial investigations concerned the rate of growth as a function of salt concentration generally. Cells were grown in multiples of the basic salt concentration (N). Figure 12 indicates that the basic medium (N) (with added urea) permits a more active initial growth than do multiples of this concentration (2N, 3N).

The growth curve in 2N medium suggests that an adaptation time is required before cell division reaches its maximal rate. Figure 12 supports this hypothesis by clearly demonstrating a more pronounced inhibition as the salt concentration in the medium increases from 2N to 3N. After sufficient time passed, cells in 2N medium divided as rapidly as those in N medium. It was therefore concluded that cells, given time to adapt, were tolerant of relatively wide variations in ionic strength of inorganic salt substrates. This is further demonstrated in Table 4.

In Table 5 final suspension densities are recorded of media with half and with twice the normal concentrations of nutrients. Substrate nitrogen was added to all media in sufficient amounts. The results indicate that the normal medium has less capacity to support cell increase than the medium with twice the concentration of salts. Apparently, one of the inorganic nutrients becomes limited with normal medium concentration.

2. Methods of Approaching the Problems Concerning a Cell's Minimal Requirements

The next logical step in the investigation was to determine the absolute amount of each ionic species required by a cell from the time of its independent existence to its division.

TABLE 4

Increase in O. D. (relative units) of media of various salt concentrations (N; 2N; 3N; see text). Respective media inoculated with cells obtained from suspensions of same ionic strength (shake-cultures)

Time (hr)	Media		
	N	2N	3N
0	100	100	100
1-1/2	150	152	150
3.0	220	250	220
4-1/2	370	390	380

TABLE 5

Final O. D. values obtained with media of various medium concentrations (1/2N; N; 2N) (shake-cultures)

Time	0 Hour	15 Hours
1/2N	0.21	5.3
N	0.21	8.5
2N	0.21	18.3

Two methods of analysis are possible. First, the cells can be grown normally in a medium and then isolated from it. Analysis of the medium before and after cell growth correlated with the increase in cell number during the growing period would indicate the amount of each element in the medium absorbed per cell. However, since such small changes in many of the elements occur (particularly micronutrients $10^{-6} \longrightarrow 10^{-9}$ M), the normal techniques of quantitative inorganic analysis are impractical. Neutron activation analysis was the second possibility considered, but even with such sensitive techniques, many of the element's concentrations were on the threshold of accurate detection. The basic elemental ratio of the medium had to be distorted to provide a proper ratio of each ingredient. Further, even after adjustment, some of the macronutrient spectra obscured some micronutrient spectra by inundation; thus, the practicality of the neutron activation analytical method appeared to diminish.

The other alternative was to grow actively dividing cells in a medium which lacked one essential element. Both Mg and Fe were tested. Growth and division would cease when the element in question was completely exhausted. A small amount of the element could then be added and observations carried out until growth ceased again. The amount of material required per cell could then be calculated.

Preliminary investigations on thin cell suspensions suggested that the rate of division does not change with sufficient speed at a point of single-element substrate impoverishment but rather gently slopes off. Studies on high density cell cultures appear to hold more promise.

3. Effects of Metals and Alloys

To assess the effects of construction materials on the development of Hydrogenomonad cultures, two alloys (SS316, aluminum) and three metals (tin, magnesium, iron) were tested. The procedure followed was to cut strips from the respective materials which were placed in the shaking cultures.

Of the materials tested, only iron showed a significant effect. Two changes were observed. First, a beneficial effect was borne out by an increase in final optical densities. The magnitude of the increase, ranging from a few % to 50% over controls, depends upon the total mass of cells produced in the medium. If final densities on the order of 5 to 10 O.D. units were considered, the increase due to the presence of metallic iron was insignificant. If the cultures were allowed to grow longer, the densities obtained in the presence of metallic iron were significantly higher. It is assumed that due to the presence of the metal, a certain concentration of ferrous ion is maintained in solution ($Fe + 2Fe^{+++} \rightarrow 3Fe^{++}$). The ferrous ion is, according to Repaske,[6] superior to the ferric ion with respect to growth support of Hydrogenomonas eutropha.

A disadvantage of the presence of the metallic iron is that a precipitate is formed. This was also observed in media with two and three times the normal

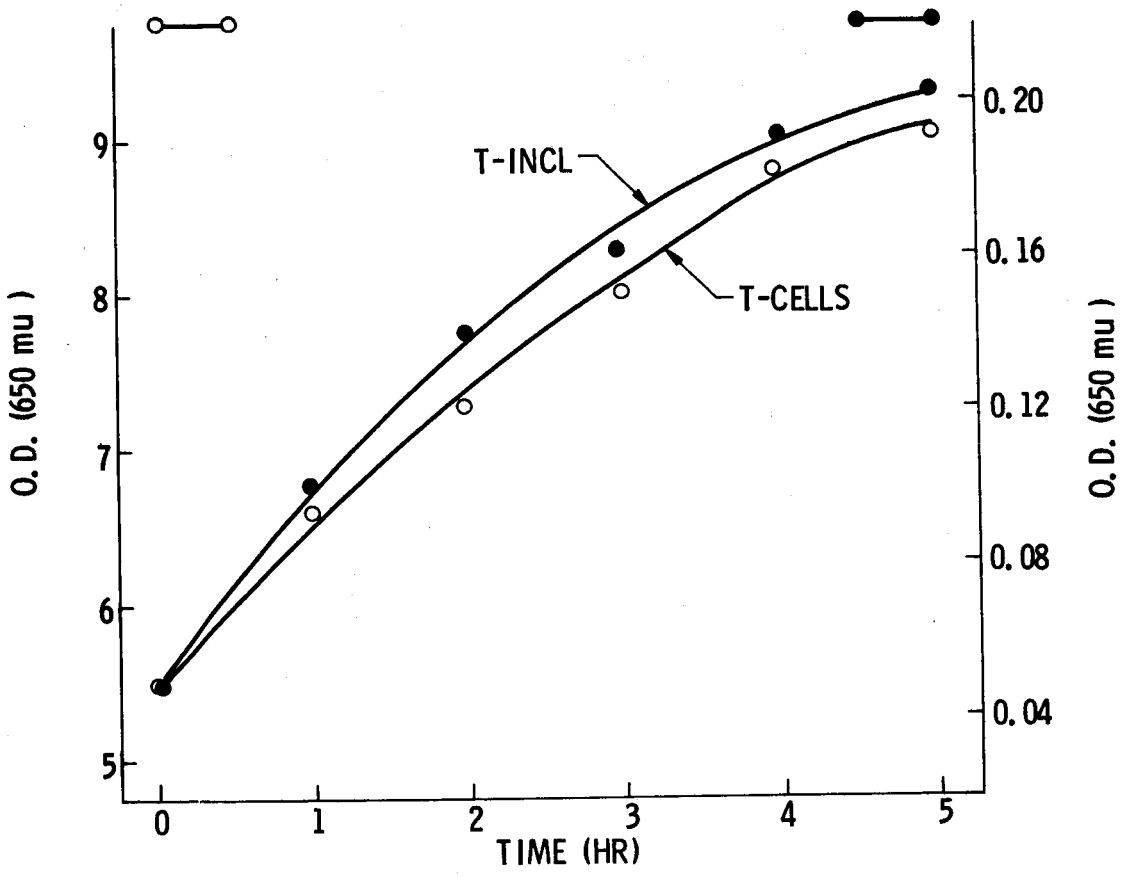


Fig. 13. Time course of N-limited growth.
T-cells: increase in optical density of the suspension (left ordinate).
T-incl: increase in the ratio of the optical density of the inclusions over the optical density of the cells (right ordinate).

concentration of nutrients. Qualitative flame spectrophotometric analyses of the sediment obtained from suspension with metallic iron showed that the precipitated material contained P, Fe, and Mg.

4. The Effect of Nitrogen Starvation

The cultural environment plays an important role on the metabolism of Hydrogenomonas eutropha. As discussed earlier (Chapter III), the biochemical distribution of the products of carbon dioxide is markedly altered by suboptimal oxygen supply. The pattern of carbon dioxide incorporation is changed even to a larger extent by deficiency in substrate nitrogen. Nitrogen starvation leads to the formation of cell material rich in carbohydrate and lipids. This phenomenon is common to many microorganisms.[13, 20 to 24] The objectives of the research efforts described below were to study the effects of nitrogen starvation on the rate of synthesis, the efficiency of energy conversion and fat accumulation in Hydrogenomonas eutropha.

When urea-grown, "lean" cells are resuspended in a nitrogen deficient culture medium and provided with hydrogen, oxygen and carbon dioxide, an increase in cell material can be observed (Fig. 13). Analyses of the cell material formed under these conditions show that the formation of lipoid inclusions starts immediately with the onset of nitrogen starvation, and is continuous throughout the time of experimentation. Isolation and estimation were made according to Williamson, et al.[10, 25]

The rate of gas uptake (per unit volume of suspension) by the cells cultured under these conditions declines rapidly to about three-fourths of the starting value, after which a constant level is maintained (see Table 2). After three to four hours of nitrogen starvation, the rate declines further.

TABLE 6

Gas Consumption Under N--Limiting Conditions Gaseous Substrate: 80% H_2; 10% O_2; 10% CO_2, Temperature 35° C; Hydrogenomonas eutropha

Gas Uptake (liters/hour)

Time (min)	Volume (liters)	Dry Weight (grams)	HyOx Fraction
0			
30	3.50		
60	3.40	1.5	.65
90	3.50		
120	3.40	1.4	.68
150	3.40		
180	3.20	1.2	.66
210	3.10		
240	3.00	1.0	.67
270	2.80		
300	2.60	0.9	.77

The decline in the specific rate of gas uptake (this is the rate of gas uptake per unit of dry weight) was observed to be more gradual during the course of the experiment. The decrease in gas consumption indicates that the biosynthetic rate per individual cell declines in the absence of a usable nitrogen source, and that the new cell material which is formed under these conditions is storage material.

The efficiency of energy conversion is constant during the first four hours of nitrogen starvation (see Table 6, HyOx Fraction). The results indicate that nitrogen starvation causes a decrease in the uptake of hydrogen, oxygen and carbon dioxide, and we may assume that the molar ratios in which the three gases are consumed remain constant during the first four hours. During this time, the nitrogen content of the cells has decreased to values on the order of 7 to 9%. This nitrogen content coincides with a decrease in conversion efficiency. Gas consumption measurements of these cells show that the rate of carbon dioxide uptake becomes proportionally lower, and, consequently, the energy of conversion becomes less efficient. With further decrease in nitrogen content (the lowest nitrogen content observed was 4% in dry weight), the carbon dioxide fixation rate declines sharply, and the gas which is consumed consists almost entirely of hydrogen and oxygen. The rate of gas uptake of cell material with 4% nitrogen is only 10 to 15% that of normal cells.

If energy conversion efficiency is considered in terms of the number of hydrogen molecules oxidized for the fixation of one molecule of carbon dioxide, then the results described above indicate a strong decrease in efficiency of energy conversion in cells with N-content of less than approximately 8%. This phenomenon can be defined as Leerlauf or idling. However, some caution is in order. The energy obtained in the hydrogen oxidation reaction in the absence of carbon dioxide fixation is not necessarily lost. As observed by Schlegal, et al.,[25] the storage of energy-rich polyphosphates can occur under these conditions, but the energy content of the polyphosphate proved rather low relative to the energy generated in the hydrogen oxidation reaction. Another way in which energy storage in the cells may occur in the absence of actual carbon dioxide fixation is the further reduction of the metabolic products present in the cell. As an example, the conversion of carbohydrate into fat requires energy, but not necessarily fixation of carbon dioxide. Energy stored by the cells in this way would escape detection by the method used for efficiency analysis in these experiments.

In Table 7, data are presented of experiments in which the effects of suboptimal oxygen supply and nitrogen starvation upon cell fattening are compared.

TABLE 7

Cell "Fattening" in Presence (I) and Absence (II) of Urea; Temperature: 35° C; Gaseous Substrate: 75% H_2; 15% O_2; 10% CO_2

Time		0 Hour	38 Hours
Dry weight g/l	I	1.90	8.5
	II	1.90	6.7
Nitrogen content (%)	I	14.0	7.4
	II	14.0	4.1
Fat content (%)	I	Trace	21
	II	Trace	44

These results indicate that a relatively higher fat content and a higher absolute amount of fat can be obtained in the absence of nitrogen. To study the nature of the lipids formed, ether-alcohol and chloroform extracts were made from cell material of different state of cell fattening. The preliminary results are shown in Table 8. The extractions of the three samples show differences in composition. To determine the significance of the variations in extractable material, further analysis of the composition of the extracts will be required. These analyses* are presently in progress.

TABLE 8

Extracts from nitrogen-starved cells (I), from cells grown under oxygen limitation (II), and normally cultured cells (III). Cells cultivated with 10% CO_2, 15% O_2, 75% H_2 in 35° C

Sample	Cell Wt	Ether/Alcohol		Chloroform		Total	
	(mg)	Wt (mg)	Percent	Wt (mg)	Percent	Wt (mg)	Percent
1	608	19.3	3.2	147.6	24.3	166.9	27.4
2	1099	51.1	4.6	108.8	9.9	159.8	14.5
3	354	35.9	10.2	7.9	2.2	43.8	12.4

*Gas-liquid-chromatography analysis of methylesters are carried out by Dr. W. T. Miller, Department of Chemistry, Regis College, Denver, Colorado.

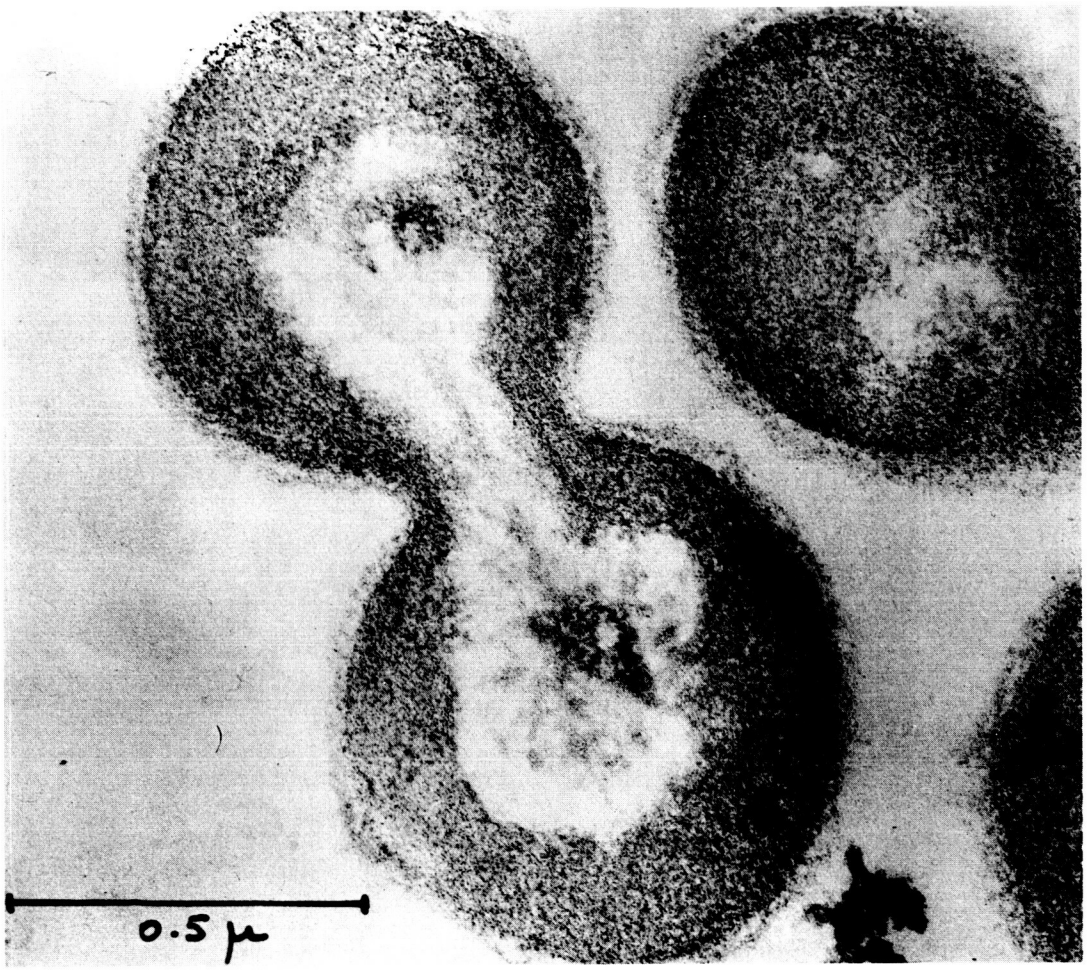


Fig. 14. Electron micrograph of Hydrogenomonas eutropha in phase of cell division

The morphological changes in the cell which upon nitrogen starvation occur are illustrated in Figs. 14 and 15. These electron micrographs represent a lean, dividing cell (Fig. 14), and a fat cell (Fig. 15) cultured in the absence of substrate nitrogen. A comparison reveals the distinct globular inclusions which are clearly distinguishable in the cytoplasma of the nitrogen-starved cell (the N-content of these cells was approximately 4%). In these fat cells, very little nuclear material can be seen. Cells in this state of N-starvation showed no cell divisions, but upon transfer to fresh normal medium, the cells start dividing again after a long time lag.

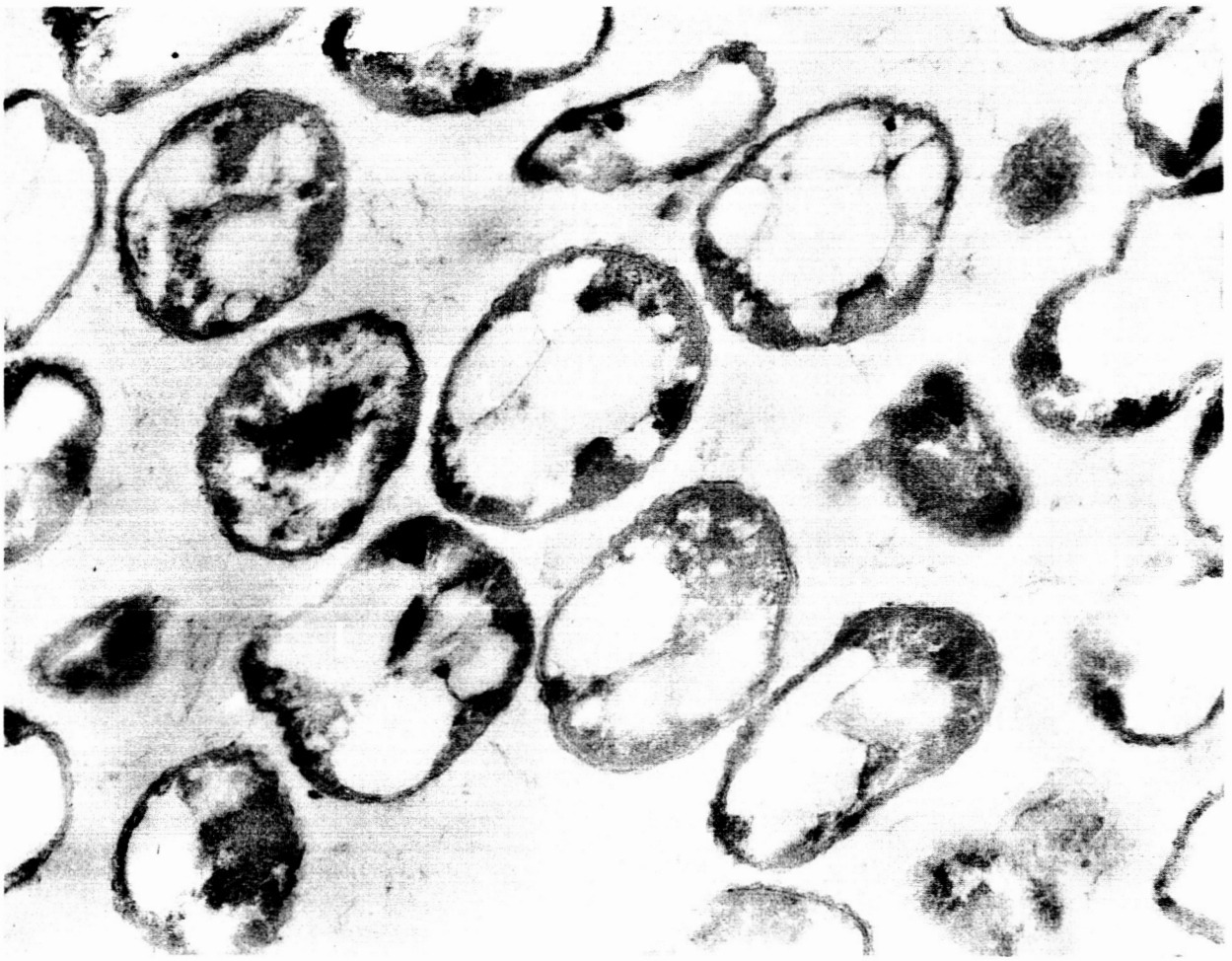


Fig. 15. Electron micrograph of Hydrogenomonas eutropha, "fat" cells. L: fatty inclusion. Cells are nitrogen starved.

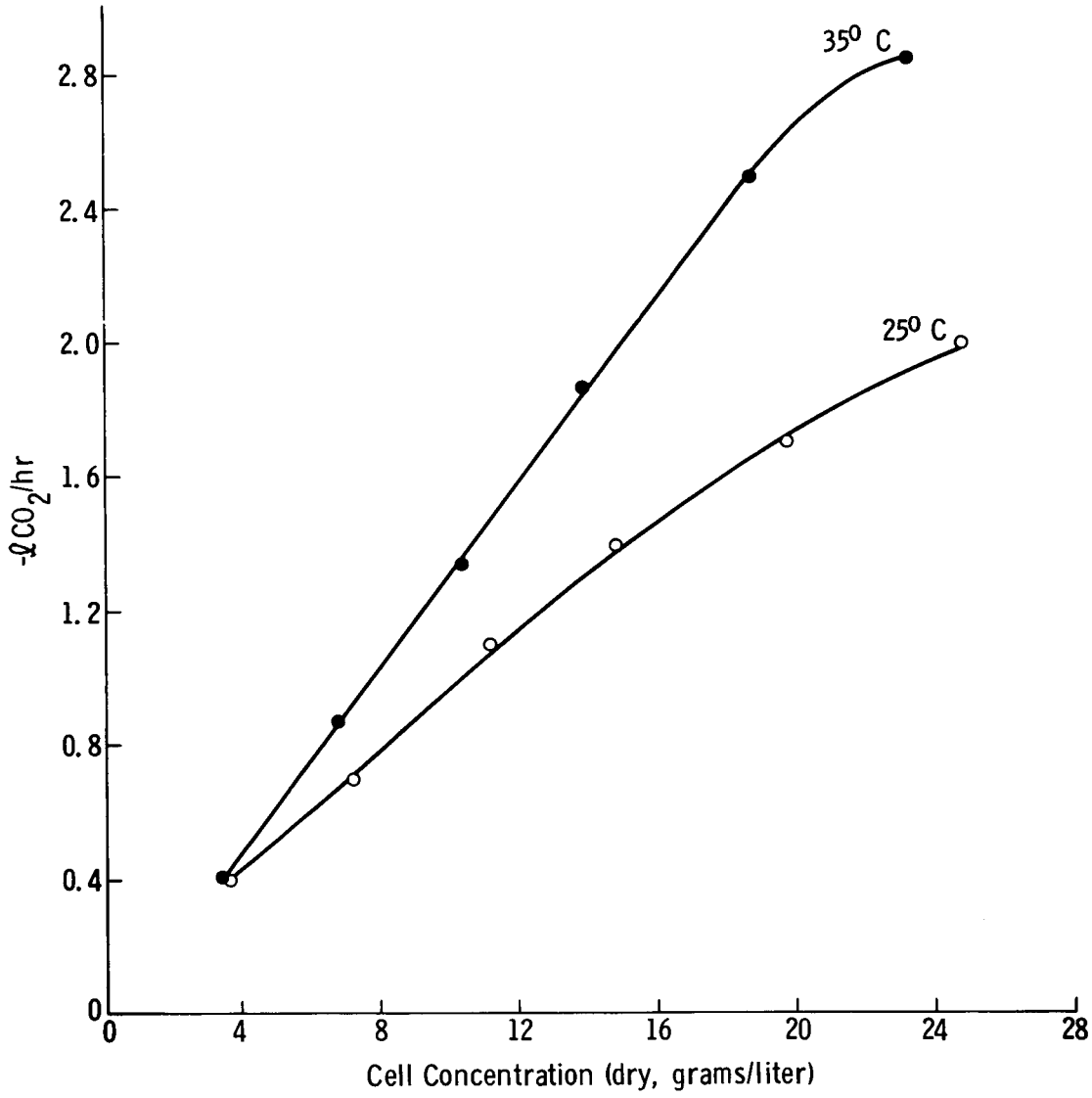


Fig. 16. Carbon dioxide consumption rate versus cell density. Hydrogenomonas eutropha. Gas phase: 80% H_2, 10% O_2, 10% CO_2. Ordinate: liters CO_2 taken up by one liter of suspension per hour. Abscissa: cell concentration, expressed as grams of dry weight per liter.

V. RELATION OF CELL CONCENTRATION, GROWTH AND EFFICIENCY

The implementation of a closed ecological system requires consideration with respect to weight (volume) and power demands. Two parameters of major importance in this respect are the cell concentration at which the biosynthetic system can be operated and the energy which is required to drive the biosynthetic process. In order to evaluate these aspects, a series of experiments were conducted at various cell concentrations and the rate of cell production gas consumption and the efficiency of energy conversion determined.

1. CO_2 Consumption Rate Versus Cell Density

Rates of gas consumption were measured manometrically, utilizing the reaction vessel illustrated in Fig. 4, and the rate of CO_2 consumption calculated from the total gas consumption. Experiments were initiated at high cell concentrations from which dilutions were made to obtain the various cell densities. After each dilution step, the rate of gas consumption was measured for a period of 20 minutes. The results thus obtained are recorded in Fig. 16.

The results indicate that at the higher temperature an appreciably larger CO_2 fixation occurs. The rate of consumption at the optimal temperature was found to vary from 2 to 2.5 liters of CO_2 per liter of suspension per hour. A linear relation between the rate of CO_2 fixation and cell concentration was observed in the density range from 4 to approximately 20 grams of dry weight per liter. The Q_{CO_2} values in this density range are on the order of 140 at 35° C and 95 at 25° C. With densities in excess of approximately 20 grams, a decline is observed which is most probably due to passing limitation.

2. Cell Density Versus Growth and Efficiency

Growth rates and conversion efficiencies were measured of cultures of Hydrogenomonas eutropha initiated at various degrees of cell concentration. The cell concentrations examined ranged from a few to 35 grams (dry weight) per liter. The cell material for these experiments was obtained from shake cultures by concentration. The sediment was subsequently resuspended in fresh media with adequate amounts of substrate nitrogen and the cell concentrations adjusted at the desired level. The suspensions were subsequently transferred to the reaction vessel pictures in Fig. 4. A gas mixture of 10% CO_2, 15% O_2, and 75% H_2 was constantly recirculated through the suspensions. The increase in suspension dry weight was determined at the start and finish of each experiment, which lasted 4 hours. The rate of gas uptake was measured at regular time intervals, and, from the increment in dry weight and from the rate of gas consumption, the efficiency was determined according to the method described earlier (see Chapter III). The results thus obtained are recorded in Table 9.

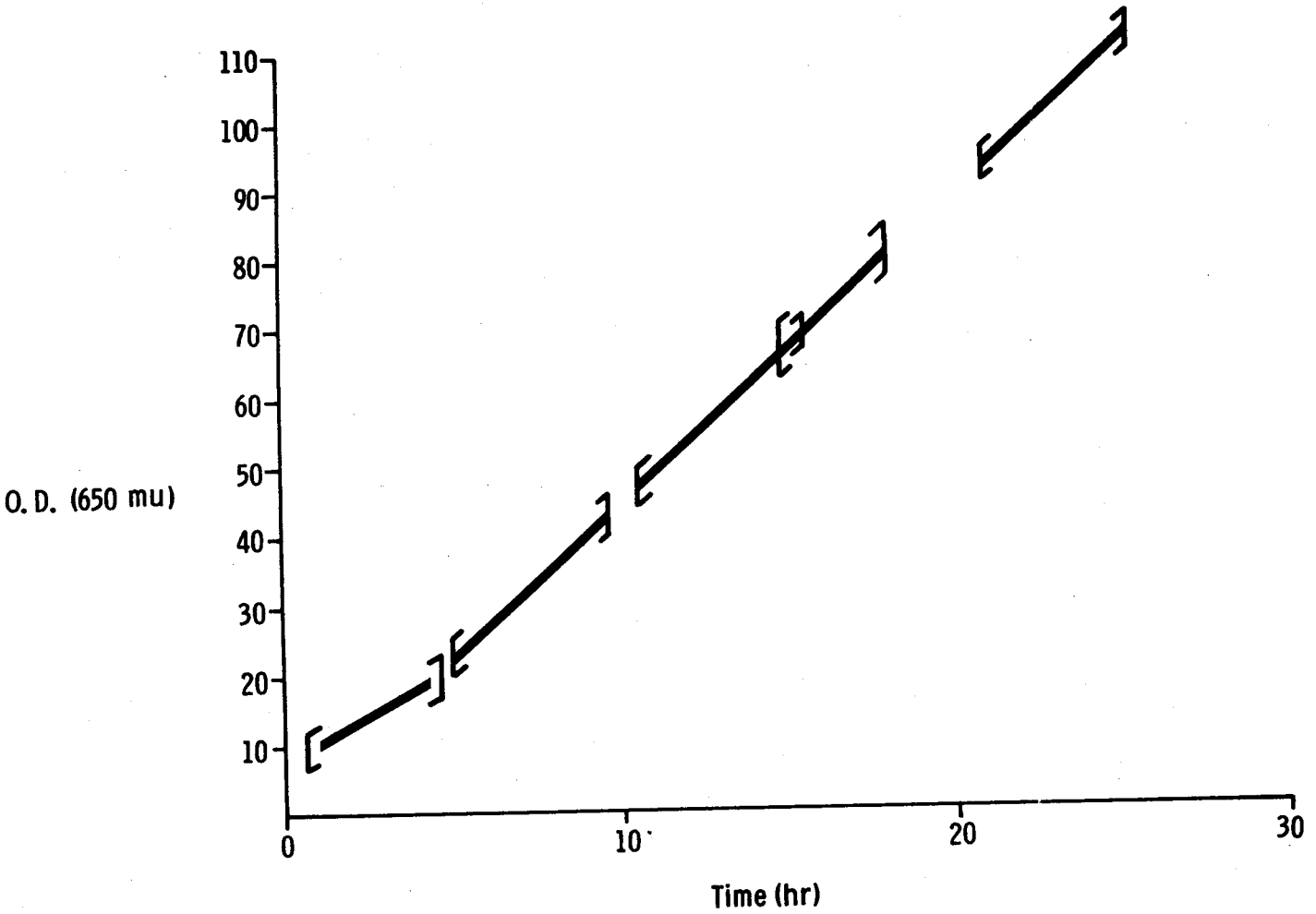


Fig. 17. Growth rates of high density cultures. Increase in culture density (measured at 650 mu) versus time measured at time intervals of approximately five hours. Gas composition 30% O_2, 50% H_2, 20% CO_2. Average growth rate over total curve about 5.3 optical density (the equivalent of 1.8 grams/liter hour) units/hour.

The data indicate that, for the set of conditions under which these experiments are performed, cell concentrations on the order of 10 to 15 grams (dry) per liter are optimal with respect to growth rate (see Table 9). Cell concentrations in excess of these values usually do not lead to higher rates.

It is presently not clear whether gassing conditions, inadequate nutrient supply or chemical changes in the environment are to be blamed for this limitation. An explanation may also be sought in the excretion of inhibitory products, the accumulation of which may directly be related to the number of cell divisions which occur in the medium. Another possibility was that cell multiplication was limited to the formation of storage material, a phenomenon discussed in Chapter III. To study this possibility, cell material formed in suspensions of high density was analyzed for globular inclusions. In these experiments (see Fig. 17), more or less identical growth responses were observed (the average rate of growth is 1.8 grams dry weight per liter per hour) as reported in Table 9, but no poly-β-hydroxybutyrate inclusions were found, not even at the highest density investigated. Another yet unknown cause must therefore induce the transition to inactivity.

The conversion efficiency is only slightly affected by cell concentration (see Table 10). On the average, the values observed with cell concentrations of 10 grams dry weight per liter or less indicate a somewhat better conversion efficiency than with higher cell densities, but the difference is statistically not significant.

The dry weight production rates, as measured over a four- to five-hour time span, represent an average CO_2 fixation rate on the order of 2 liters per hour, similar to those observed in short term experiments (see Fig. 16). If these rates can be duplicated under continuous cultivation conditions in a functional unit, the suspension volume required is on the order of 10 to 15 liters per man.

TABLE 9

Effects of cell concentration on growth rate and conversion efficiency. Suspension temperature 35° C; gas phase: 10% CO_2, 15% O_2, 75% H_2

Average Cell Concentration		Growth Rate	Dry Weight Increase (4 hr)		Efficiency
g/l	OD (650 mu)	g/l hr	g/l	OD (650 mu)	$N \frac{HyOx}{CH_2O}$
5.9	20	1.2	4.7	14	3.4
6.1	20	1.5	5.1	15	3.9
6.7	22	1.0	4.0	12	3.9

TABLE 9 (continued)

Average Cell Concentration g/l	Average Cell Concentration OD (650 mu)	Growth Rate g/l hr	Dry Weight Increase (hr) g/l	Dry Weight Increase (hr) OD (650 mu)	Efficiency $\frac{HyOx}{N}$ CH_2O
6.9	23	1.1	4.4	13	4.7
7.5	25	1.3	5.0	15	5.0
10.5	31	1.9	7.5	22	5.0
12.1	34	1.7	6.8	20	4.4
14.0	42	1.8	7.2	21	4.3
15.8	47	1.6	6.4	19	5.0
25.4	76	1.9	7.7	23	5.5
28.5	82	2.8	11.5	34	5.2
31.1	104	2.2	8.7	26	5.9
32.4	108	1.9	7.7	23	5.2
32.9	117	1.8	7.1	21	5.2

SUMMARY

The effects of temperatures ranging from 20 to 42.5° C on the growth rates of Hydrogenomonas eutropha were examined. The optimal temperature was found to be 35° C. Experiments at 25° and 35° C indicated that the efficiency of energy conversion was essentially identical at both temperatures.

Hydrogenomonas eutropha requires, as part of its substrate, a mixture of three gases--hydrogen, oxygen, and carbon dioxide. Experiments were performed to determine the toleration limits of ratios of the gases in the mixtures. Nitorgen was used as a balance. Identical growth was found when hydrogen varied from 5 to 80%. Nearly identical growth was obtained when carbon dioxide partial pressures were 5 to 60%, being slightly lower at higher partial pressures. The organism was highly sensitive to oxygen. Solvated oxygen concentrations above 0.13 millimolar were found to inhibit cell division; concentrations of oxygen below this level induced the formation of the storage material poly-β-hydroxybutyrate. Energy utilization at 0.20 mM oxygen concentration was found to be approximately twice as high as at 0.05 mM oxygen.

A second substrate requirement for this organism (aside from the gas mixture measured above) is an aqueous solution of inorganic salts. Toleration to salt concentration was examined. Growth was found to be unaffected, after an adaptation period, by three times basic concentrations. Introductory experimentation on minimal salt requirements have been performed. The effects of several metals and alloys on growth were investigated. The effects of nitrogen starvation were examined with regard to cell products and the efficiency of energy conversion.

Efficiency of energy utilization and growth was measured in a series of cell cultures of different densities. While efficiency showed no statistically different values between light (approximately 10 O.D. units) and heavy (100 O.D. units) suspensions, growth was unexpectedly constant over the range of densities examined.

RECOMMENDATIONS

A closed ecological system containing man balanced by Hydrogenomonas eutropha demands that our knowledge concerning these bacteria be extended. Consideration of the problems has indicated that future efforts be directed along the following lines:

(1) More exact quantitative evidence concerning the inorganic nutrient requirements of the cell.

(2) Circumstances surrounding the demands of a continuous culture and factors which might be detrimental to continuous, active, growing conditions.

(3) Initiation of an investigation of the actual biochemical content of the whole cell, both in terms of nutritional value and metabolic plasticity (the production of "fat-like" material under controlled circumstances could be a significant advantage considering the relatively high protein content of the actively growing cell with respect to human nutrition). Cell walls and other normally nutritionally intractable material must also be considered and carefully analyzed.

(4) The possibility also exists that the present strain of bacteria is not ideal for our specific application. Strains whose optimal growth temperature is higher would, for example, be technically advantageous.

It is believed that crotonic acid is a metabolic precursor of poly-β-hydroxybutyrate. If crotonic acid is more satisfactory nutritionally, the accumulation of the polymer would be a disadvantage.

Possibly, strains now exist which do not have these disadvantages and they should be sought. Further, irradiation of the present strain, and proper screening of mutants might well produce advantageous types. Both of these lines of investigation will be pursued.

(5) Methods of harvesting, cell preservation, and processing require further investigations.

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